

British universities not yet free

The financial pressure on British universities has been eased in the past few weeks. But there is a great deal to be done before the universities and the government are reconciled.

GOVERNMENTS are constitutionally incapable of confessing that they have been wrong. That is the most charitable explanation of how the British government, having declared its intention of being beastly towards British universities, is now trying to undo some of the damage it has done not by the simple device of reversing earlier decisions but by inventing entirely different policies instead. In the second half of 1980, the then new government decreed that students at British universities from outside the European Community would have to pay "economic" fees, and that the annual subvention for British universities would be reduced by 8.5 per cent over the succeeding three academic years. But now the government has set about undoing these arrangements. At the end of last year, the Secretary of State for Education and Science announced that roughly a third of the reduced budget cut would be restored, principally by encouraging universities to apply for new teaching posts, ostensibly so that they could recruit as academics some of the young people who have been kept out of the university system by the privations of the past several years. And then, last week, the Foreign Secretary (no less) announced that there will be an extra £30 million a year to help support overseas students at British universities and other institutions of higher education. This is getting on for a half of the income lost to the university system in the past few years as overseas demand has responded to market forces. But the extra funds will be used (in a way not yet specified) to help recruit overseas students from particular parts of the world — British dependencies such as Hong Kong, and countries such as Malaysia which have made a fuss.

So can British higher education — polytechnics and colleges of further education as well as universities — now relax secure in the knowledge that the government has relented? Not, it is to be hoped, for a moment. The "new blood" money is an admission that the system as a whole could be permanently damaged if the recruitment of young people, virtually at a standstill for the past five years, were still further delayed. But the new teaching posts to be created — perhaps 300 in the coming year, up to a thousand later on — will not be in the gift either of universities or of the University Grants Committee. Instead, the research councils will have a hand in deciding which applications for extra posts should succeed, while there is no assurance that universities successful in the competition for new blood will concomitantly be allowed some relief from the iniquitous student quotas which at present compromise their freedom and constrain their efficiency.

The concession on overseas students is even more of an unknown quantity. The government's objective is frankly political — to foster (or to repair) relationships with communities overseas that will yield long-term benefits to Britain. That governments should bias programmes of overseas assistance in ways like this is proper, even prudent. But, by definition, such a programme will not select those students whose presence at British universities and polytechnics is most desirable on educational grounds. Nor will it necessarily moderate the often unseemly scramble in which British universities have sought to maximize their incomes by recruiting students from overseas without scrupulous regard for their qualifications — and for the likelihood that they will benefit from the educational experience for which they are required to pay. What the universities need to fight for now is the government's recognition that even students

contribute to the well-being of educational institutions, and that there are many circumstances other than those defined by the Foreign Office in which it is to everybody's advantage that overseas students should be helped to attend British universities. The Committee of Vice-Chancellors is administering a modest scheme along these lines, but needs more money to spend.

As things are, the universities are badly placed to argue this or any other case. The British government persists in its belief that the university system is effete, and that there is still some way to go before the system is as economical and as productive as it should be. Inevitably, the response of the university system to the demands made on it is muffled, and seems grudging. But the year ahead will be filled with ructions unless some progress is made on these important issues:

- **Student grants.** The government has announced an increase for the value of these mandatory awards of only 4 per cent for 1983-84, thus rudely preempting the case the Committee of Vice-Chancellors had been preparing for a more generous increase. That one consequence will be hardship for many students is undeniable, especially because British students are less able than those elsewhere to supplement their incomes by vacation work. Yet the universities themselves remain indifferent to the government's declared need to find some way of containing the cost of student maintenance within financial limits that can be predicted in advance. The present stopgap remedy, the requirement that the university system should reduce the numbers of British students on its books, is arbitrary, leads to the waste of teaching capacity and should not be tolerated. But the universities will be able to escape from it, or some yet more fiendish arrangement, only by devising some better scheme of their own.

- **Academic tenure.** The concept of life-long tenure for established academics, widely misunderstood, has become an issue between the universities and the government. The vice-chancellors have dutifully drawn up an awkward set of proposals for the modification of tenure, the most conspicuous feature of which is that there should be an eight-year probationary period for all permanent appointments. This is both a recipe for throwing large numbers of would-have-been academics onto the labour market at an inconvenient age and a needless restraint on universities' freedom to manage their own affairs. In the long run, it would be far better to aim at smaller academic staffs, and to make fuller use of postdoctoral people and even graduate students as teachers. But even the vice-chancellors' scheme has not yet been agreed among the universities.

- **Applied research.** Part of the government's recipe for making British universities more productive appears to consist of the earmarking of research and development funds for particular fields. What is called information technology seems to head every list of priorities, including that put forward at the end of last year by the Advisory Board for the Research Councils. Another large chunk of support for the same activity seems to be on the way from the Department of Industry (see page 646). The obvious danger is that the pattern of research in the university system as a whole will be unwisely distorted by these initiatives, however well intentioned. A more serious difficulty stems from the suspicion that the British government has only a naive concept of what it wants, and that experience may compel it to change course. Who, within the university system, will speak up on this contentious issue? □



TV cable in chains

Unless the British Government thinks again, its promised revolution in cable TV will come to naught.

THE British Government's cable revolution will not now take place. Almost exactly a year ago, the cabinet's advisory panel on information technology declared that Britain must be swiftly rewired for 30-channel cable television or face the consequences: missing out on the information age. Until then the industry minister, Mr Kenneth Baker, had been so blind to the virtues of cable-TV that he had launched his Information Technology Year and its accompanying glossy pamphlet without a mention of cable. But immediately the advisory panel's report was out, he giddily promised that cable-TV would cure every kind of ill, from unemployment to educational apathy among the young.

The doubts began to set in in November. The Hunt report on cable, which was supposed to reconcile British broadcasting traditions with multichannel television, did no such thing. It merely said that cable did not need the kind of regulation that was appropriate to over-the-air broadcasts. Mr Baker, for his part, took note of the arguments that cable television might not be the stepping stone to the future electronic grid if it were laid out in the tree-and-branch system used in North America and on the mainland of Europe. (This feeds signals along a trunk cable from a base to the subscriber's home. Each home gets all the channels carried by the cable and needs a filter to select the one it wants or has agreed to pay for.) He promised that cable systems in Britain would be laid in a star pattern. The trunk cable carries the full array of signals only to a local hub. Then the cables travelling into homes carry only three or four channels, obviating the need for a complex decoder. Because the hub provides a local switching point, like a telephone exchange, this system offers the possibility of being upgraded later into a telephone or video phone system.

In December, Mr Baker told the House of Commons that British cable operators would be required to lay their cables in the star pattern. If they used conventional (coaxial cable) technology, they would have only a 12-year licence. If they invested in the even more adventurous fibre-optic cable, they could have a 20-year licence. Then he, the potential investors and the public sat back to wait for the promised white paper from the Home Office, still the final arbiter of broadcasting policy.

The deadline is slipping. The Home Office is labouring to produce it knows not what. All the irreconcilables still remain to be reconciled. Which authority should regulate cable franchises, fix the franchise areas and decide what social obligations should be laid upon operators? How to protect British broadcasting standards from a flood of channels filled with whatever subscribers are willing to pay for? How to preserve national televised sacred moments like Wimbledon tennis and the Grand National for broadcast television? How to keep children out of the adult-film channels which cable systems can provide for willing subscribers?

In the year since the cabinet panel reported, new fears have surfaced. Mrs Margaret Thatcher is terrified of introducing televised pornography into the British home (and knows that pornography suits cable, financially and technically). Worse terror has been struck in the hearts of the City of London's institutional investors by the likely sums required for cable-TV, British-style. If cable must be buried underground, if potentially unprofitable areas must be wired along with the more desirable, and if they have to invest in the star pattern, which is of no economic use in delivering home entertainment, there is no way they can get their money back in less than ten years.

Ten years? By then the British television audience may have disappeared entirely. One of the shocks of the past year has been the disappearance of 10 per cent of the usual audience. Whether they have gone to sleep, into the garden or to the video film rental shop is the subject of much heart-searching in the higher reaches of the British Broadcasting Corporation and commercial television companies, but also among would be cable-sellers. If the public is fed up with four channels plus breakfast television, will it want to pay the high cost needed to hook onto cable?

In the light of all the new difficulties, the government should stop and think again about a curious ban it has placed upon the cable television service — provided new systems ever get built at all. It is that cable systems may use their cables for the two-way interactive services (they are the goal of this entertainment-led information revolution, after all) but that they may not use them for telephony. Voice telephone traffic, and presumably viewphone when its time comes, are to be the protected preserve of British Telecom and its fledgling competitor, Mercury Communications. So what is the point of requiring cable systems to invest in star systems that are capable of being upgraded into a telecommunications service? The government must make up its mind.

Either cable television should be allowed to expand purely and simply because British consumers are entitled to the extra channels of television which technology can now provide. Or Britain desperately needs a national broadband information grid capable of carrying high-speed data and videophone into the far corners of the land. If the first, cable investors should be allowed to build the pattern of system they choose. If the second, they should be able to use it for voice — and to take British Telecom on as a partner wherever possible. The government is silly to expect private investors to pay more than they need to build a system whose logical technical capacities they are prevented from using. But investors are not silly, they are selfish. That is why the cable revolution is not going to take place. □

Small step forward

Belatedly, the United States plans to ratify two arms control treaties neglected for the past decade

THE United States Government has at last done the sensible thing about the two arms control treaties which have been hanging around unratified for the best part of a decade. Last week the Administration let it be known that it plans to blow off the dust from the Threshold Test-Ban Treaty (1974) and that on Peaceful Nuclear Explosions (1976), take up some questions bearing on verification with the Soviet Union and then put the treaties to the US Senate for approval. This modest and long-overdue development will have three distinct advantages: First, it will give both superpowers the benefits of the two treaties which they have been hitherto denied — the exchange of seismic information near test-sites provided for under the Threshold Test-Ban Treaty and the right of on-site inspection specified under that for regulating peaceful nuclear explosions. Second, it may provide an opportunity for each side to install on the other's territory remote seismic sensing stations of the kind planned during the Comprehensive Test-Ban negotiations, aborted in 1980. Third, it will go some way to show that the US government is not entirely indifferent to the importance of arms control — the impression that has been created in the past few weeks by rigidity about the "zero-option", the firing of Mr Eugene Rostow, the dismal showing of his nominated successor and the complete lack of a response to what Mr Andropov has been saying, most notably in the declaration put out after last month's meeting of the Warsaw Pact.

But what real purpose will be served by ratifying two treaties which appear to have been faithfully observed by the two superpowers since they were first negotiated, and which have made no noticeable difference to the pace of the accumulation and deployment of nuclear weapons? It is true that ratification will seem strictly cosmetic to the members of the Non-Proliferation Treaty (whose next review conference is due in just over two years), who will be looking for a more tangible proof that the superpowers have kept their promise to negotiate "substantial" measures of nuclear disarmament. For that, success in the negotiations now under way at Geneva, or a comprehensive test-ban is necessary. The obvious snag is that a ban on all nuclear tests will be unattainable until there is some progress on limiting the deployment of nuclear missiles, which requires not merely that West Germany should have gone to the polls (on 5 March) but that some agreement should have been reached on the British and French nuclear forces. So ratification of these two forgotten treaties is the best hope for 1983. But something is a lot better than nothing. □

Data falsification

NIH decrees ten-year ban on research grants

Washington

THE National Institutes of Health (NIH) recommended last week that Dr John Darsee, a former Harvard Medical School research fellow found to have fabricated data, be barred from receiving federal research funds for the next 10 years.

NIH will also ask Harvard's Cardiac Research Laboratory at Brigham and Women's Hospital — which was held indirectly responsible for lax supervision that "may have contributed inadvertently to the ease with which he was able to produce fabricated data" — to repay the \$122,371 that NIH provided for the project whose results Darsee doctored.

The action came after an NIH-appointed panel of experts investigating Darsee's work at Harvard found a pattern of data fabrication. In addition to the falsified data in the NIH-supported project, the investigators found "statistical aberrations" in five papers that Darsee published while at Harvard which "cast doubts upon the primary data". These papers have since been retracted by Dr Eugene Braunwald, the chairman of Harvard's department of medicine.

Although Darsee will have 30 days to appeal against the decision to the Secretary of Health and Human Services, he has already acknowledged, in a letter to the NIH staff, that the investigation had established the falsifications and his role. He claimed to have no recollection of falsifying any data.

Suspicious were first aroused about Darsee in May 1981, while he was working on a heart study in dogs. Several laboratory technicians observed Darsee labelling strip-chart tracings obtained during a single day "24 hours", "72 hours", "one week" and "two weeks". The next day, when Darsee presented these results to his supervisor, Dr Robert Kloner, he was confronted and admitted his guilt. Braunwald subsequently withdrew an offer of an assistant professorship at Harvard and asked Darsee to resign his NIH fellowship, which had been supporting him at Harvard since his arrival in July 1979. But Braunwald and Kloner decided not to inform NIH of the reason. They also allowed Darsee to continue work on the NIH-supported project that became the focus of the investigation.

Braunwald explained to NIH in December of last year that "what was known in May 1981 was that a promising researcher with a long history of achievement had committed and acknowledged a single act of misconduct . . . Dr Darsee insisted that this act was a single incident".

Suspicious turned to alarm in October 1981, when the preliminary results of the NIH-supported study were compiled. The

results obtained by the Harvard group turned out to be inconsistent with results obtained by four other groups doing similar studies as part of the same project (known as AMPIM). Dr Kloner then decided to reveal the May incident. Kloner also discovered a serious difference between Darsee's results for this study obtained before the May incident and after, when he was more closely supervised.

The NIH investigation began in December 1981, and, in a report completed last summer (but only made public last week), confirmed that Darsee had fabricated the AMPIM results and earlier results as well. Within the past three months, evidence from Emory University, where Darsee worked before coming to Harvard, has called into question more of Darsee's publications. According to

Braunwald, who has contacted Emory's internal investigation, co-authors of abstracts submitted while Darsee was at Emory may not even have been aware that Darsee was listing them as such. "The integrity of six published papers, three of which involved experimentation with human subjects, is in question, as are a large number of published abstracts", Braunwald stated in a December 1982 memorandum to NIH on the Emory investigation.

The NIH investigation recommended that although supervision at the Cardiac Research Laboratory has been tightened up, NIH should inspect the laboratory to ensure that procedures to prevent a repetition of the problem are adequate. Braunwald and Kloner fought unsuccessfully, against NIH's adopting this recommendation.

But the investigators noted that part of the blame lies with the way research is done in the modern research laboratory: "A hurried pace and emphasis on productivity, coupled with limited interaction with senior scientists, has contributed to the disappointing events." **Stephen Budiansky**

Weapons research

Stanford jibs at military contact

Washington

A PROPOSAL to use Stanford's Synchrotron Radiation Laboratory for a weapons-related research project has met widespread opposition from the faculty and staff of the adjacent Stanford Linear Accelerator Center (SLAC).

Nearly half of SLAC's faculty last month signed a letter opposing the project, which was proposed by Livermore, Los Alamos and Sandia National Laboratories along with the University of California. Part of the project would involve the use of synchrotron X rays to calibrate X-ray detectors used in weapons tests.

"We've never had any weapons-related research here", said Dr George Loew, one of the 15 signatories of the letter, which was sent to Stanford University's Committee on Research. "It's a departure from the main mission of the laboratory. We asked whether the university could develop some policy so this work and any future work of the same nature wouldn't be done."

Although Stanford has a strict policy forbidding classified research on campus, it has no policy on unclassified but military-related research. The Livermore project would be completely unclassified, and only a portion would involve weapons applications. As submitted to Stanford, the proposal calls for construction of a new beam line to be shared equally between the weapons laboratories and the University of California researchers. None of the University of California researchers would be involved in the weapons-related research. The Department of Energy's office of military applications has been asked

to provide \$5.2 million; the University of California investigators would provide about \$1 million.

The proposal faces several hurdles, however, and seems a long way from approval. It is being considered by the synchrotron laboratory's proposal review panel, a group of outside experts that examines all proposals for their scientific merit. The panel has asked the Livermore group for more details.

If the review panel clears the project, it would still need the approval of the synchrotron laboratory's director, Professor Arthur Bienenstock. The synchrotron laboratory is administratively separate from SLAC, but uses electrons produced by the accelerator.

A third hurdle is a Stanford requirement that outside projects should have a Stanford principal investigator. "It's possible nobody [at Stanford] wants to be associated with that work", Loew said.

Lloyd Multhaupt of Livermore said that the project is "primarily basic research", and that "only a small portion of the proposal" involves the X-ray detector calibration. He also said that "the military side of the Department of Energy has funded a lot of basic science". Multhaupt said that the national laboratories were also looking into the possibility of carrying out part of the project at Brookhaven National Laboratory's synchrotron source.

Loew said that he and other opponents to this project are not objecting to weapons research *per se*, but they believe that a university campus is not the place for it.

Stephen Budiansky

Biotechnology

Troubled company back in credit

Gaithersburg, Maryland

BETHESDA Research Laboratories Inc. (BRL), the prominent US biotechnology firm that hit trouble a year ago after growing rapidly since it was set up in 1976, is thinking of making a public stock offering. M. James Barrett, the former Smith-Kline Beckman executive who was made BRL's president as part of the rescue operation, says that the company now has enough "real value" to go public some time in the next year.

BRL's renewed interest in offering shares for public sale may be influenced by the very strong recent market in biotechnology stocks (*Nature* 10 February, p.459). The strong market may also have caused another biotechnology-based company, the Swiss Biogen NV to apply to the Securities and Exchange Commission to make a public offering. Centocor, a smaller firm in Malvern, Pennsylvania, went public in December.

Some Wall Street analysts are sceptical as to whether BRL has changed from its high-flying days which nearly led to bankruptcy (*Nature* 296, 3; 1982). Only the eleventh hour intervention of venture capitalist Frederick R. Adler saved the company. Adler arranged emergency financing, almost exactly a year ago, on the condition that he be given complete control as chairman of the BRL board's executive committee.

In the reorganization, the president Stephen C. Turner, who founded the company was made chairman of the board. Turner and Adler recruited James Barrett

from Smith-Kline Beckman. Barrett's reasons for believing BRL to be healthy once again shed light on the inner workings of the many biotechnology firms springing up in the United States.

BRL was founded by Turner in 1976 to capitalize on the new need for enzymes and reagents among researchers in the then-new field of genetic engineering. BRL's sales grew, along with its reputation as a principal supplier of materials and instrumentation for genetic engineering research. In 1977 sales stood at \$350,000. In 1980 they stood at \$5 million.

The phenomenal growth, just when biotechnology was becoming fashionable in the financial world, encouraged BRL to look to further expansion by raising funds by a public stock offering. This caused it to diversify into 11 different "hot" areas.

Fundamentally, the research and development programme was designed so that the prospectus (the booklet of promises a company issues when it offers stock for public sale) would show "page after page of enormous technology potential residing at BRL", Barrett says. With this approach, the company raised \$16 million in private stock offerings over two years.

But the market for BRL's products could not support such rapid expansion and at one point BRL had only \$800,000 per month coming in from sales and was spending \$2 million per month. The stock market was in the doldrums in early 1982, so hopes that the company could suddenly acquire cash from the long-sought public stock offering faded. By early 1982, some \$9 million in back debt had accumulated. The crisis broke and Adler stepped in.

BRL's retrenchment cost hundreds of employees their jobs (the firm now employs 214, down from a peak of around 450), the breaking of a contract to buy a large building and the quick sale of Glenco, a company acquired to take BRL into the field of chromatography.

Barrett laid off more people and cut out activities not central to BRL's core business, so as to get a positive cash flow. Although Adler raised \$9 million to pay off back debts, BRL did not have a positive cash flow until last November.

Ironically, BRL was saved in part by the growth of the rest of the biotechnology field: despite all its problems, sales in 1982 reached \$13 million — \$3 million more than in 1981. The projects cut were programmes in instrumentation development, cell biology and the use of growth factors in therapy. BRL had a lot of work in diagnostics but has stopped all but some long-term research in cancer and viral diagnostics.

Barrett sees BRL as remaining a principal supplier to the research community, but branching into a few carefully selected "end user" areas.

Deborah Shapley

Information technology

Prospect of more research money

THE British Government seems to be moving towards acceptance of the chief recommendations of the Alvey report on information technology, published last autumn. In a report to the Department of Industry, a committee under Mr J. Alvey, a director of the telecommunications monopoly British Telecom, urged a five-year programme of research and development costing £350 million, three-quarters of the cost to be met by the government and the rest from industrial sources.

Mr Kenneth Baker, the minister at the department with special responsibility for information technology, told the House of Commons last week that he would "soon" be able to announce the government's response. The proposals have not yet been considered by the cabinet, which is likely to be especially concerned with two features of the proposals — that three-quarters of the total cost should be met from public funds, and that the programme should be managed by a special directorate set up within the Department of Industry.

Meanwhile, funds to support some parts of the programme appear already to have been allocated. Last year's earmarking by the Advisory Board for the Research Councils for the support of information technology is indeed more than that recommended by Alvey, at least in 1983–84, but in future years the Science and Engineering Research Council will have to divert funds from elsewhere in its budget if the Alvey target is to be met. Similarly, the Department of Industry's research and development budget for the coming year has been increased by £60 million to £218 million, more than the cost of meeting the obligations spelled out by Alvey even after allowing for inflation.

The Alvey report seems to have won the hearts and minds of British ministers by its emphasis on the need to strengthen basic technology in Britain. The intention is to concentrate on four areas of research — software engineering, very large scale integration of circuits, what is called the man-machine interface and "intelligent knowledge-based systems". The report also includes a specific timetable suggesting when particular goals in each of the four chosen fields should be attainable.

One of the most remarkable features of the report is that its authors were able to promise on behalf of the British Ministry of Defence that military interests in the development of information technology would be coordinated under the same umbrella, although projects related to particular defence needs would continue separately. There are few precedents for this apparent readiness to acknowledge that defence needs are not unique.

New blood flows in

THE University Grants Committee is this week sifting through more than 1,000 applications for the 200 new university posts at British universities made available under the scheme for recruiting "new blood" in science, engineering and medicine. Although the closing date for applications was passed last Friday, applications were still flowing in earlier this week.

Altogether, there are 300 new posts available, 30 in the humanities and the social sciences while 70 are earmarked for specialists in information technology, more than half of whom will be employed in running training courses of various kinds. The present plan is that applications for the 200 places in science, engineering and medicine will adjudicated by the end of March by the grants committee and the research councils, when successful universities will be allowed to advertise their vacancies.

The scale of application appears to have varied dramatically, with some universities applying for only a handful of new posts and other asking for close on a hundred. □

Siberian mammoths

Gone to the dogs

A GROUP of Siberian construction workers who recently fed their dogs with meat from a frozen mammoth that they had unearthed, instead of reporting their find to the proper authorities, have caused a major shock to Soviet public opinion. Mammoths, to the average Russian, play a particularly emotive role in the country's



prehistory; well into the nineteenth century, some optimists still believed that a living herd might yet be located in the remote wastes of Siberia.

The locals, however, seem to be more blasé. During the 1920s several cases were reported of travellers being offered — and on occasion eating — mammoth flesh. Even if these stories are all untrue, an export trade in mammoth ivory flourished

from the eighteenth century until Soviet rule was fully established. (It has been estimated that during this time the tusks of 48,000 mammoths were exported.)

In the case of the most recent find, when, too late, the on-site geologist tried to remonstrate with the men, they merely shrugged and remarked that this would not be the last mammoth to turn up in Yakutia.

The workgang was engaged on construction for the gold mining industry — a somewhat ironic link since, as *Pravda* pointed out last week, a whole preserved mammoth would fetch \$100,000 on the world market. (The reward offered last century by the Imperial Academy of Sciences for a well-preserved frozen mammoth was a mere 500 gold rubles.)

The conservation and exhibition of well-preserved mammoth specimens has always been a major concern of Russian palaeontologists — the classical examples being the complete specimen brought from Wrangel Island in 1938 and the 1977 find of a baby mammoth, which formed the highlight of the Soviet exhibition in London in 1979.

Professor Nikolai Vereschagin, of the Institute of Zoology of the Soviet Academy of Sciences, however, has an even more ambitious idea — the recreation of the mammoth by genetic engineering, using cloning techniques to produce, first a semi-hairy hybrid, and subsequently a mammoth. So far, however, none of the frozen mammoth carcasses examined by the Academy's Institute of Cytology has yielded revivable cells. The chagrin over the latest find being used for dog-meat is doubtless all the greater.

Vera Rich

Japan's second X-ray satellite launched

JAPAN seems set to take the lead in X-ray astronomy with the successful launch of the ASTRO-B satellite by the Institute of Space and Astronautical Science (ISAS) on Sunday 20 February. The satellite, named *Tenma* (Pegasus) joins *Hakuchō* (Cygnus), launched in 1979 and still functioning, to give Japan a monopoly in X-ray astronomical observation until the launch of the European Space Agency's EXOSAT later this year.

Tenma's most important detector is a gas scintillation counter which has not previously been flown on a satellite and which has doubled the energy resolution of the usual proportional counters. With an area of 1,000 square centimetres — five times that of the gas scintillation counter to be carried on EXOSAT — it will allow spectral analysis of low-intensity X-ray sources and make it possible to classify many of the sources seen in previous satellite missions.

Tenma also carries a Hadamard transform telescope specially designed to scan wide areas of the sky for high intensity radiation from X-ray bursters. Japanese astronomers have taken the lead in this field since *Hakuchō*, launched not long

after the discovery of bursters by Massachusetts Institute of Technology scientists, was successful in finding eight new sources. A soft X-ray detector and a small gamma-ray burst detector are also on board.

The X-ray astronomical programme will continue with the launch of ASTRO-C in 1986 or 1987. Possible participation by the United Kingdom in the project is being considered in discussions with ISAS.

Japan is unique among space nations in that its scientific space programme is run entirely by the universities and is completely separate from the main programme, aimed at launching commercial telecommunications, navigation and resource satellites and largely using imported US technology. The two groups do not even share launch sites. ISAS, until recently a part of the University of Tokyo but now a separate institute providing facilities for all universities, has developed its own solid-fuel rockets and has launched 13 scientific and test satellites since 1970. It consumes about a tenth of the total space budget, now running at a little over 100,000 million yen (£250 million) annually.

Alun Anderson

European Space Agency

Bias claim

THE European Space Agency (ESA) will decide on its next space mission on 26 March, when the Science Programme Committee will choose one project for "phase B" development. Although the recommendations have been agreed by the Space Science Advisory Committee, geophysicists involved are complaining that the project concerned with geophysics and planetology, the Kepler Mars orbiter, has been voted down because of a built-in bias towards astronomy and astrophysics within ESA's committees.

The five missions originally considered are a solar "seismology" satellite DISCO, Kepler and the satellites ISO, Magellan and X-80, designed for infrared, ultraviolet and X-ray astrophysics respectively. The Solar Systems Working Group (SSWG) was asked to rank DISCO and Kepler for scientific merit and chose DISCO. The Astronomy Working Group (AWG) carried out a similar study of ISO, Magellan and X-80 and chose the first.

DISCO, designed to measure oscillations in solar spectral lines caused by waves in the solar interior, is said by SSWG to "exploit ... a novel field of astrophysics". The working party says that the Kepler mission would allow "a wide community of European scientists" to study a variety of problems, that there is no overlap with previous US and Soviet satellites and that Kepler is a "unique and valuable opportunity of exceptional scientific merit, deserving full support".

In spite of this accolade, SSWG recommended DISCO. The Space Science Advisory Committee has gone one step further and has recommended ISO rather than DISCO on the grounds that it would be a timely successor to IRAS (the infrared satellite launched earlier this year) with a substantial increase in sensitivity and spectral resolution which would also give valuable experience of supercooled technology in satellites.

The Science Programme Committee, which will make the final selection from the five projects on 26 March, will not be bound by the advice from the scientific working groups. Indeed, at least one member of committee is hoping that it will not be. Marcel Ackerman, of the Belgian Institut d'Aeronomie Spatiale, feels that some of the potential achievements of ISO are overstated in the advisory committee's report and questions whether the technical experience is readily available in Europe to cope with the cryogenic technology required for ISO.

He also raises a broader issue. DISCO, although concerned with the Sun and therefore within the SSWG's remit, is nevertheless essentially an astrophysical project and should therefore, according to Professor Ackerman, have been considered along with the other three

astronomical projects. He adds that because the working party includes scientists whose primary interest is solar physics, the dice are loaded towards astronomy and away from solar system geophysics.

Professor Ackerman points to the United States while making this charge. In his view, it is symptomatic of the contrast that Europe is incapable of coordinating an effective campaign such as that in the United States to investigate the atmospheric effects of the El Chichón eruption. He points to the Solar Mesosphere Explorer (SME) satellite as an important element in that programme, quoting it also as an illustration of how the US National Aeronautics and Space Administration allows scientists greater independence to manage their own budgets, thus avoiding the bureaucracy of ESA.

If the space agency chooses ISO, then, with Giotto and Hipparcos already under way, no ESA mission will be planetological in orientation. "The decision will have far-reaching consequences" says Professor Ackerman. "On a longer time scale, some political decisions are going to have to be made at the highest possible level if Europe is to keep at least some contact with others in the realities of space."

When told of Professor Ackerman's complaint, Dr A. Gabriel, chairman of SSWG, said that the Kepler proposal was a good one, well-judged in cost and scope for an ESA project, and that he was surprised that it had not won more support. But he indicated discreetly that not all members of the working group with planetological interests were united behind the project.

Philip Campbell

Heath care in London

Improved plans

A MUCH-DELAYED step towards the improvement of health care in Inner London was taken last week by the University Grants Committee, which has set aside £160,000 to help found departments of general practice at two London teaching hospitals — the partnership of St Bartholomew's and the London Hospital Medical Colleges and St Mary's Hospital Medical School. This is almost the first step taken to implement the recommendations of Professor Donald Acheson's study group on London's health care, published nearly two years ago.

The essence of the Acheson report's diagnosis of health care problems in Inner London was its complaint that there had been inadequate progress (up to 1979) towards the development of group general practices in which patients are looked after by integrated health care teams. The provision of adequate care for patients in Inner London had been complicated in the 1970s by the redistribution of acute hospital beds away from London — one of the chief reasons for the committee's existence.

Surveys carried out by the study group at the end of 1979 drew attention to the difficulties encountered by new patients in finding physicians who would accommodate them on their lists.

Many of the Acheson recommendations take the form of financial incentives and disincentives to improve the organization of general practice. Carrots include the suggestions that physicians should be paid extra for each new patient registered, extra payments to encourage the merging of practices or to salaried assistants intended to be made full partners in a practice. The corresponding sticks include a revision of the rule that a physician should be able to claim from the National Health Service his rent and rates and 70 per cent of the salary of ancillary staff provided that he or she has more than 100 patients on his books, and of the rule that other public contributions towards overhead costs should be calculated on a sliding scale based on 1,500 patients per physician, not 1,000 as now.

Many of these recommendations have disconcerted individual physicians working in Inner London, while physicians elsewhere have seen it as a threat to their standard terms of service. For the past six months, the government has been under pressure in the House of Commons to say when its response would appear, while even the General Medical Services Committee of the British Medical Association is "seriously dismayed" at the delay. The University Grants Committee, one of the members of the London Health Planning Consortium that commissioned the Acheson study seems, however, to have responded early to the plea that facilities for professional training and retraining should be improved. □

Federal elections

What the parties promise

Canberra

SCIENCE and technology feature prominently in the election platforms of the two principal Australian parties campaigning for election on 5 March. What follows is a list of the promises made by the Australian Labour Party (ALP) and by the Liberal-National Party (LNP) which formed Mr Malcolm Fraser's government.

The Australian Research Grants Committee (ARGC) at present spends A\$18 million a year on academic research projects, the National Health and Medical Research Council (NH and MRC) has a budget of A\$33 million a year and the Australian Industrial Research and Development Incentives Scheme (AIRDIS), administered by the Australian Industrial Research and Development Board, now costs about A\$50 million a year.

Vimala Sarma

Australian Labour Party

Increase ARGC funding by 10 per cent over inflation rate for the next 3 years.

Initiate national research fellowship scheme for post-doctoral research, costing A\$2.3m in the first year for 100 fellowships.

Make AIRDIS grants tax-free.

Convert present Australian Industrial Development Corporation into Australian Industrial Development Bank to provide venture capital for high-technology industries. (Priorities identified — computers, computer software, custom-made chips, scientific instrumentation, medical technologies, lasers, communications technology, industrial ceramics, solar technology, shape memory alloys, fusion, robots, hydrogen as fuel, biomass.) Establish new research, development innovation division within DST.

High technology

Increase NH and MRC funds over current inflation rate by A\$4m in 1983–84, A\$8m in 1984–85, and A\$12m in 1985–86.

A\$46.5m for establishing an advanced technology corporation to assist firms and organize venture capital with a ceiling of A\$100m for the first year. Make approved projects 100 per cent tax deductible.

Biotechnology

Make funds available through AIRDIS, the new Australian Development Bank and a new industry finance corporation.

Establish a national biotechnology scheme for funding basic as well as applied research, costing A\$2.5m in first year increasing to A\$15m in third year.

A\$2.5m from AIRDIS grants.

Antarctic research

Increase funding by 300 per cent over 3 years. Provide aircraft landing facilities.

A\$4m for improving transport facilities.

Australian Telescope

Honour Fraser government's initiative.

A\$25m over 5 years as a bicentennial project.

Starlab

Provide funds.

Supporting phase B studies.

Conservation

Stop the Gordon-below-Franklin dam by use of existing constitutional powers.

A\$500m compensation offer still open.

Provide funds for alternative projects.

A\$50m to establish a national park in south-west Tasmania.

Establish environmental contaminants authority.

A\$5m for a national soil conservation programme.

A\$4m for a national soil conservation programme.

Other projects

Establish an international prize awarded by Australian Academy of Science of the value of the Nobel Prizes for scientific achievement in human welfare.

Upgrade Landsat receiving stations.

Underground nuclear tests

Reagan seeks ratification of treaty with Soviet Union

Washington

PRESIDENT Reagan has decided to negotiate a protocol with the Soviet Union strengthening verification provisions in two treaties on underground nuclear explosions signed by both countries in 1974 and 1976 but never ratified by the United States.

Senator Charles Percy (Republican, Illinois), chairman of the Senate Foreign Relations Committee, announced on 16 February that the White House had informed him that negotiations could start "in a matter of weeks" if the Soviet Union is willing. The decision implies that the President does not want to ratify the treaties as they are, a goal sought by Percy and several other members of the Senate.

Percy had asked the President's nominee as director of the Arms Control and Disarmament Agency, Kenneth L. Adelman, whose nomination is in trouble, to try to break the logjam that has delayed the Administration's ruling on the treaties for two years. Although they are technically already before the Senate for ratification, the Senate is unlikely to proceed without support from the White House.

Percy has been concerned about the two treaties since his visit to Moscow in 1980 on taking over as Foreign Relations Committee chairman. On his return, he announced that ratification of the two treaties by the Senate was important to maintain US credibility with the Soviet Union on arms control. The more important agreement on strategic arms, Salt II, signed by both sides, has also never been ratified by the United States.

The Threshold Test Ban Treaty (TTBT) and the Treaty on Peaceful Nuclear Explosions (PNE) were negotiated in the Nixon-Ford era and signed in 1974 and 1976 respectively. They prohibit either side from detonating underground nuclear blasts of any kind over 150 kilotons in yield and are to be verified primarily by the seismic detection nets that detect distant tremors in the Earth and distinguish earthquake signals from those of large explosions.

The PNE treaty has an added attraction to US arms controllers in that each side agrees to some form of on-site inspection. Since combined explosions of more than 150 kilotons are allowed provided advance notice is given to the other side, the other side may then verify with its own equipment on site to be sure that no single explosion in the group exceeds the 150 kiloton limit. The clusters feature was included in the PNE treaty because of Soviet interest in large earth-moving projects.

Since the treaties were negotiated, however, each side has accused the other violations. On twelve occasions, US monitors have recorded tremors that could

have originated from blasts of more than 150 kilotons, but detection limits and seismic calibrations are not sufficiently refined to be sure.

The Soviet Union has denied that any of the tests exceeded the treaty limit and claimed that on five occasions blasts at the Nevada test site in the United States exceeded the limit. The United States denied these charges. Whether such claims and counterclaims would sour a future negotiation over verification of the treaties is not clear. Neither is it clear why the President, after two years of delay, has decided to renegotiate. One reason given by Administration officials is that the history of violations proves that existing provisions are inadequate.

On the other hand, the opponents of arms control often criticize arms control agreements on the grounds that verification is inadequate. These people could have agreed to the idea of renegotiation as a way to avoid having to ratify the treaties.

The most hopeful possibility is that the two sides will agree to incorporate in the verification system for the threshold test-ban treaty the system of up to ten automatic seismic stations on each other's territory agreed upon in 1980 as part of the comprehensive test-ban treaty. Technically, these stations would have broken new ground by their use of public-key cryptography as a means of giving each side access to the data collected by the other.

Any reopening of the test-ban treaty through talks with the Soviet Union could run into another problem, the modernization of inter-continental ballistic missiles (ICBM). Many Soviet warheads have yields above 150 kiloton, and Soviet weapons-builders could well take advantage of a hiatus in the treaties to test these higher yield weapons. Last week there were reports that the Soviet Union had tested a second new ICBM. Under Salt II, each side is allowed to test only one new ICBM type.

Besides the two test limit treaties, senators on the Foreign Relations Committee are also interested in reviving negotiations towards a comprehensive test-ban treaty. President Carter had put the two treaties on a back burner while trying instead to complete a comprehensive test ban. But the test ban negotiations were broken off in 1980 after the Soviet invasion of Afghanistan.

A resolution introduced in the Senate by Edward Kennedy (Democrat, Massachusetts), Charles Mathias (Republican, Maryland) and others urges the Administration to resume negotiations on a comprehensive test ban. It also urges the Administration to ask the Senate to ratify the two nuclear test limit treaties.

Deborah Shapley

French research

Contract work

Sophia-Antipolis

FRENCH researchers may have to take an examination half-way through their careers, if they wish to advance to a higher post, according to a new "contract of employment" being prepared by the Ministry of Research and Industry in Paris.

This requirement is a step towards meritocracy and democracy in French science — or so its perpetrators claim. It will help separate advancement by age and seniority from advancement by merit, and provide a relatively uniform standard by which researchers can be judged.

An outline of the new contract was presented here last week at a conference on French science policy by Claude Kordon, himself a researcher. Kordon is not a politician but despite his protests that the new contract not completely defined, it is clear he is flying the government's kite.

The contract, says Kordon, would divide researchers employed by the principal French research organizations into two classes. In each class, a contract of employment would have two components: one guaranteeing "minimal career", offering a slow growth of salary with age, and another allowing acceleration along the scale according to "evaluation of work".

The lowest class would end at the present *maître de recherche*. Examination would allow transition to the top class, which would end at the present highest level of *directeur de recherche*. The allocation of these top grades would be done by the Minister of Research and Industry himself "to avoid any devaluation of the title". He would be advised by the Conseil Supérieur de la Recherche et de la Technologie.

The only dangers, argued Kordon, were that too many careers would be accelerated, thus destroying the objective of the structure; or that too few senior jobs would be created to allow sufficient acceleration.

One problem is that since last year's law on research, the job of a researcher has been extended to include not only research but also science popularization and general education, and the application of his or her work. But no criteria have been established to judge these functions, or the relative weight they should take compared with research itself. So the question of how to evaluate a researcher has become even more difficult.

The second problem lies in the fact that the new career structure will separate the evaluation of salary from the evaluation of title — and from the offer of positions. There will thus be three evaluation systems running in parallel, some local and some central (based in Paris), plus an exam. A similar system, but this time with three classes of career and two examinations, will operate for technicians and senior technicians (engineers).

Robert Walgate

Belgian universities

Preparing for seven lean years

Brussels

BALANCING the books in the field of education and other areas of public spending is still causing problems despite Belgium's seven-year plan to bring expenditure into line with income. Last October (see *Nature* 299, 767; 1982), the government demanded of Belgium's universities that they should present plans to bring their growing debts and deficits under control by 1989 or risk losing their autonomy. Since then, though, the government's stance towards public spending has toughened as it became increasingly clear that despite ruthless austerity measures the public spending deficit was still out of control.

The hard line taken by the centre-right government lead by Wilfried Martens was reflected in the decision made at the end of January to reject as inadequate the plans drawn up by two of Wallonia's largest universities, Liège and ULB (Université Libre de Bruxelles).

More disquieting have been the unpleasant measures taken against Belgium's im-

of being one of the most generous European countries. Even after the French universities began closing their doors under Giscard d'Estaing, there were no limits on the numbers of students from developing countries, who enrolled almost exclusively at the francophone universities where they were seen as a useful way of boosting income. Under the Belgian system, the state funds universities according to the number of students registered.

But more tussles between the education minister, Michel Tromont, and some of the universities can be expected in the coming week. The education minister decided at the end of January to reject the plans of the universities of Liège and ULB. They have been given until 15 March to come up with more satisfactory proposals. ULB, with some 40,000 students one of Belgium's largest universities, is asking for BF 500 million (£6.6 million) which it claims is still owing to it from a previous administration and has taken the government to court. The ministry remains intransigent and demands that the university redoes its sums.

Liège, with 10,000 students, is in the deepest trouble. Faced with an accumulated debt of BF 1,000 million (£13.3 million), its seven-year plan was rejected because it still allowed for a post-1989 deficit of around BF 700 million. A state university, as opposed to ULB or the Catholic University of Louvain-la-Neuve (UCL), which are free universities, it has about 300 unwanted ancillary staff who have the status of civil servants and therefore cannot be fired. Liège will have to find another way of reducing its costs.

Many other Belgian universities have been forced to delve into their *patrimoine* or assets accrued from carrying out research, from gifts or by selling off land or buildings. Professor Woitrin, general administrator of UCL, feels that the ways being used by Belgian universities to solve financial problems of the sort being faced all over Europe can serve as useful lessons to others. Woitrin's own university has cut the number of ancillary staff by 250 and has negotiated an agreement with all employees to cut the gross wages bill by 15 per cent through a progressive reduction in salaries. The staff will in real terms be accepting a drop in spending power of up to 4.25 per cent for the highest paid. In return they can expect to have their loss in earnings matched by increases paid once the seven lean years are over.

This leaves UCL and other Belgium universities more favourably placed to face the future compared with government-controlled institutions in France, Germany and the United Kingdom. Cuts in teaching staff have been avoided and the numbers and mobility of research assistants have also been unaffected, although a restructuring of courses is in hand. **Jasper Becker**

Education in China

Government seeks reform

SWEEPING changes in the structure of higher education in China, advocated by a recent forum in Beijing (Peking), would give the university authorities considerable autonomy over admissions, employment and financial policy. The minister of education, He Dongchang, who addressed the symposium, said that the reforms were "imperative" and should be carried out "with the strongest resolve".

The proposed package of reforms would give universities and higher colleges the right to dispose of funds earned by their research and consultancy work for industry and allow lecturers to take on part-time work outside.

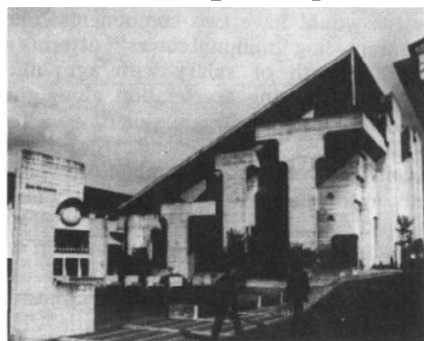
The forum particularly condemned the practice of sharing out the wage fund equally, regardless of individual effort and performance. Under the new system, students would be encouraged to be more adventurous in their studies, dropping their present assumption that, once admitted to a university, they are assured of a job for life.

The minister emphasized the need for a reform of university management which, to judge from the Chinese press, is urgent. Until six months ago, for example, the "educational and administrative leadership" of Hunan University included only one person who had received a university education.

Minister He specially commended the Chinese University of Science and Technology and the Jiaotong University in Shanghai for their management reforms. In the past two years the latter has cut its lecturing personnel from 1,040 to 700, at the same time increasing the volume of teaching work by 30 per cent, and raising its earnings from research and consultancy work for industry from 800,000 yuan in 1981 to 1.5 million yuan in 1982.

The intended reforms aim to enable the universities to meet the demands of the "four modernizations" that are the keystone of Chinese development policy. The value of university-trained specialists is not, however, always clear to the Chinese rank and file who still show the anti-intellectual bias inculcated during the Cultural Revolution.

The Chinese media and party propagandists are mounting a major campaign against this attitude, both with straightforward exhortation and anecdotes, such as the recent case of engineering designer Wu Bauxin. He was denounced by a group of workers at the factory where he was employed for having received a payment of 1,020 yuan for a single design, which subsequently earned for the factory 450,000 yuan. The critics, the *People's Daily* commented, "do not understand the hardships of research and design work". **Vera Rich**



The Catholic University of Louvain la Neuve, where the staff has accepted salary cuts.

migrant communities. A fortnight ago the mayor of Schaerbeek, one of Brussels' communes, closed down nine schools attended by 3,000 children, mainly from the large community of migrant workers. Roger Nols, the mayor or *Bourgmestre*, declared that the cost of supporting the schools was too high and demanded that the state intervene.

The government has been unsympathetic. It has itself taken an aggressive stance against foreigners. The children of foreign students as well as anyone without a work permit are now to be charged school fees for attending school above the official leaving age of 14. Only under considerable pressure did the government reverse its decision at the end of 1982 to oblige all Belgian universities to demand fees from all foreign students instead of leaving it to the university's discretion. Only postgraduate students from a reduced list of developing countries could hope to have their education fees paid but now the richest universities can still accept foreign students over the 2 per cent of places reserved for students from developed countries. In the past, Belgium has had the reputation

"Yellow rain" or natural toxins?

SIR — We would like to comment and expand on the feasibility of naturally occurring mycotoxins in Southeast Asia (*Nature* 23/30 December 1982, p.679). According to one US State Department supporter, the feasibility is "absurd" because, on the basis of feeding trials with cows, serum levels of 10 to 100 parts per 10⁹ T-2 toxin require massive oral doses, which presumably would not occur in the foodstuffs of alleged victims of "yellow rain". This logic is somewhat convoluted inasmuch as a metabolite of T-2 toxin, HT-2, is detected in serum samples of animals several days to weeks after ingestion, not T-2. The presence of T-2 toxin in serum samples collected several weeks after "yellow rain" attack¹ is inconsistent with previous animal studies. This inconsistency has generated the theory that human metabolism of T-2 toxin is fundamentally different from animal metabolism and is characterized by long-term "tissue binding"¹. But, if human and animal T-2 metabolism are different, then it is illogical to discount the natural occurrence of T-2 toxin by comparing T-2 serum levels in experimental cows with levels in human victims. In our view, the identification of trichothecenes in the tissues of Southeast Asians may be the result of natural mycotoxin contamination of foodstuffs.

The State Department maintains that trichothecene mycotoxins² and trichothecene-producing fusaria³ do not naturally occur in Southeast Asia. But, a T-2 toxin producing *Fusarium* species was described in the 1930s in Vietnam and Canadian investigators recently reported the isolation of trichothecene-producing fungi, such as *F. semitectum* and *F. sporotrichioides*, from "most" samples collected near the Thailand-Kampuchea border⁴.

The latest State Department report¹ argues that the detection of trichothecenes in several vegetation samples is particularly persuasive since "*Fusarium* is not a leaf pathogen". This is simply not true. *Fusarium roseum*, *F. nivale*, *F. poae*, and *F. tricinctum*, all trichothecene-producing species, have been described as leaf pathogens. Also, other genera of leaf pathogens are well known trichothecene producers. The mycoflora of the State Department's trichothecene-positive vegetation samples was not examined.

According to the State Department, mixtures of trichothecenes in certain vegetation and soil samples "are not normally found in nature and it would appear that these mycotoxins found their way into the environment by the intervention of man"¹. Numerous laboratory studies do indeed indicate that the mixtures detected cannot be attributed to the activities of any individual isolate. However, simultaneous mycotoxin production by multiple species of *Fusarium* is a well-known natural

phenomenon (see refs 5,6).

Ironically, as the State Department continues to press its case, a natural occurrence explanation of the data becomes more credible. Analyses of tissue samples of an alleged "yellow rain" victim revealed high levels of trichothecenes and aflatoxins, *Aspergillus flavus* toxins¹. Reported symptoms of the victim were consistent with aflatoxicosis and trichothecene poisoning induced by consuming moldy grains.

The military use of chemical weapons is despicable. Moreover, some of the evidence to date suggests the use of trichothecene weapons in Southeast Asia and merits intense investigation. However, the State Department's shrill accusations of Soviet treaty violations are premature. Instead of continually advocating theories of Soviet wickedness, alternative hypotheses such as natural occurrences need to be openly explored. For example, mycoflora of physical samples from attack sites and the levels of aflatoxins in all tissue samples need to be tested. Until then, scepticism will remain.

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Beware cloned toxins

SIR — No doubt a number of plant and bacterial toxins (ricin, diphtheria and so on) will soon be cloned and expressed in bacteria. The large interest in toxins is due to the hope that when attached to antibody molecules these "immunotoxins" will be a new generation of magic bullets to destroy cancer cells, fight parasite infections and manipulate cells of immune diseases, transplantation, and so on. This is laudable but a potential Pandora's box is about to be opened. It is my fear that these toxins could be used for military purposes. Just 10 µg or less can kill a 70 kg human being and a soldier hit by a toxin shell would soon die because of the difficulty of making specific antidotes to the large number of available toxins. The use of these weapons and their proliferation should be highly controlled and banned by international law, such as that banning chemical and germ warfare. I would encourage all governments to strive to have existing treaties on chemical and biological warfare amended to include these substances.

JAMES W. LARRICK

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Leuven's universities

SIR — The news item "Belgian universities hit trouble" (*Nature* 299, 767; 1982) contains a number of errors about the Katholieke Universiteit Leuven which I would like to correct.

The Catholic University of Leuven, during the 1960s, was split into a complete and autonomous Dutch-language university, the Katholieke Universiteit Leuven (KU Leuven) and a complete and autonomous French-language university, the Université Catholique de Louvain (UCL), both with their own juridical status. That is not to say, however, that the lectures were not divided and taught both in Dutch and in French previously at Leuven — such duplication of lectures began in the 1920s and was virtually complete by 1935 when the Catholic University of Leuven became officially bilingual (Dutch and French).

When the split of the Catholic University of Leuven was officially complete, in 1970, it was decided that KU Leuven would remain in the Dutch-language (Flemish) sector of Belgium, in Leuven itself. UCL was to move to the French-language part of Belgium (Wallonia) and build a new university at Ottignies, "Louvain-la-Neuve". UCL's move was completed at the end of the 1970s, and it is now established in its new buildings.

The caption accompanying the photograph of Leuven's famous university library published in the *Nature* article, therefore, is incorrect, ignoring UCL's move. The Central University Library on Ladeuzeplein is in fact part of the Dutch-language university, KU Leuven.

Moreover, contrary to what was implied in the *Nature* article, when KU Leuven has a vacancy in the academic or scientific staff and cannot fill it with a qualified Catholic scientist, it is sufficiently broad-minded to appoint a non-Catholic who does possess the required scientific qualifications. Indeed, non-Catholics have already been appointed to posts in some specialities at KU Leuven, for example, in biology in the Faculty of Sciences.

J.P. GROOTAERS

(Director)

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A sticky problem

SIR — Professor F.E.G. Cox is mistaken in his *News and Views* article on Chagas' disease (*Nature* 23/30 December 1982, p.685); it is peanut lectin which is specific for terminal galactose residues. Peanut butter agglutinin, to which he refers, is specific for the roof of your mouth, agglutinating it to either (1) the floor of your mouth, or (2) biscuits, depending upon the nature of the experiment.

J. JOHN COHEN

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Oncogenes—no to reductionism?

SIR — Your *News and Views* annotation on "Oncogenes, reductionism and all that" (*Nature* 3 February, p.369) to my mind obscures an anterior debate. Holism and reductionism are modes of thought, the recognition of which is useful so that their more extreme manifestations can be subject to criticism, which, incidentally, seems to consist of gibes in *Nature*. Most scientific endeavours have little consciousness of operating in either fashion but, to an outside observer, follow a course between the extremes. At times pressures from outside the academic community, "Rothschild" for example, can temporarily distort the pattern by asserting that there is some purpose in scientific endeavour and here, to my mind, lies the nub of the matter.

Working in an institute of cancer research one is sensitive to the criticism (not gibes, note!) that from the point of view of cost-effectiveness little seems to have been accomplished. One's colleagues on the other hand often argue that of course we need to know more before any good effect can derive from the large cost. Whether we should learn more by application of holistic or reductionistic method is not usually debated in those terms but the balance which is struck between clinically orientated and fundamental research is the outcome arrived at by drift rather than design. Thus, to my mind, the proper argument is not whether the scintillating new discoveries of the molecular biologists, manifestations of reductionism if you like, are futile, but whether science has a purpose. If it does we should almost certainly set about it in a different way.

A.J.S. DAVIES

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SIR — The *News and Views* article "Oncogenes, reductionism and all that" (*Nature* 3 February, p.369) is a little unfair to the reputable biologist (discredited vitalist or not) who would receive the welcome news of the recent contribution molecular biologists have made to the cancer problem with reserved scepticism. Cancer research is in an exciting phase, where there are now the means to understand cancer in its proper biological context for the first time. The ultimate aim of cancer research, which I would define as removing the intellectual humiliation and relative clinical impotence which afflicts clever people faced with a common disease, is already proceeding by generalization towards the elimination of that definition of cancer which sets the disease apart from all others, and in some way apart from the biology of living organisms as a whole.

Cancer can be defined as an abnormal relationship between the cellular contents of a malignant tumour and the host in which it originates, such that the normal processes of growth control and differen-

tiation do not occur either because of a primary cellular defect within a component of the neoplasm or because of an intrinsic or acquired abnormality of the host. There is no known universal constitutional defect of a cell which can, without reference to the host, be considered *a priori* to confer the properties of neoplastic growth. The normal function of oncogenes is not known, hence the impact of the oncogene in biological terms cannot be fully assessed.

It is amazing that one probably minor and insignificant biological role of the oncogene (that of causing cancer) is developed into the state where the naïve reductionist might believe that it was inserted into the genome by a jocular deity to cause the specific disease for which more than modest research funding is readily available. The importance of these findings, in relation to cancer, cannot be ignored, but neither can the work on differentiation, genetics, immunology and epidemiology. The theorist (or the author of the *Nature* article) who believes that in the light of present knowledge it is possible to objectively debate the "first cause" argument in support of the oncogene in cancer hypothesis is misinformed.

The real advance lies in the fact that we may shortly achieve enough knowledge of the cancer state to set out the ground rules for such an objective debate. As an unashamed and (I hope) reputable biologist, why need I be castigated for holding the view that currently the oncogene hypothesis is naively reductionist?

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Thalassaemia

SIR — Orkin *et al.* in *Nature* of 23/30 December 1982¹ elegantly demonstrated that decreased synthesis of mutant globin β^E chains is due to abnormal processing of the β -specific mRNA primary transcript. This explains the previous finding of decreased mature mRNA in Hb E reticulocytes². Thus, a qualitative change in globin structure in associated with a quantitative change in its production. This is a vindication of the "structure-rate" hypothesis proposed by Itano a quarter of a century ago³ and further elaborated by Ingram and Stretton⁴. At that time it was suggested that an electrophoretically silent or a synonymous codon replacement might be present in β -globin in β -thalassaemia. If this entailed the requirement for a tRNA species absent or scarce in erythroid cells, it would explain why the polypeptide chain was synthesized at a reduced rate⁵.

Apart from the special case of Hb Lepore, the search for a structural abnormality in the β -globin chain in β^+ -thalassaemia has since failed. However, we

now have a clear example of a β -globin chain which was already known to be structurally abnormal, and in which a reduced rate of synthesis (β^+ -thalassaemia) is indeed a direct result of the structural abnormality. It turns out that the link between structure and rate is not at the stage of translation, but rather at the earlier stage of processing of the primary transcript.

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Dürer's technique

SIR — Nobody who could recognize a truncated rhombohedron when he saw one would have doubted that this is the polyhedron shown in Albrecht Dürer's drawing "Melencolia" (1514) and recently discussed in *News and Views* by Philip Ritterbush¹. The only questions relate to its proportions. Assuming a threefold axis of symmetry the proportions of the solid object before projection could have been recovered by stereogrammetric techniques, as described by H.C. Longuet-Higgins². However, the whole drawing (and others by Dürer) has been analysed by E. Schröder, as to the techniques used for the projection, in the light of Dürer's own manual of geometry, "*Underweysund der messung*. . ." (1525)³.

Schröder shows that in Dürer's rhombohedron, projected in the special orientation with its threefold axis vertical, the diagonals of the faces of the untruncated rhombohedron are closely in the ratio $\sqrt{3}$ to 2. Thus, the rhombohedral angle α is 82° to an accuracy which excludes the possibilities of the golden section, which would need $\alpha = 72^\circ$ and the nearest rhombohedron of calcite ($\alpha = 76.1^\circ$). The c/a ratio is thus $3/2$. Moreover, the overall height of the object is equal to the horizontal diagonal of the faces.

Thus, it seems that the polyhedron is simply an exercise in accurate draughtsmanship and that the art historians have made rather heavy weather of its explanation. The integral proportions show that no particular mineral was intended, although Grigoriev and Shafranovskii^{4,5} had concluded that the polyhedron was an octahedron of fluorite ($\alpha = 60^\circ$) but that the figure represented had $\alpha = 72^\circ$.

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Chemicals and genetic damage

The US National Academy of Sciences has told the Environmental Protection Agency how to regulate chemicals causing inheritable mutations. But the more urgent need is for more research.

CAN CHEMICALS cause inheritable mutations? the simple answer is "Why not?". For it is well known that chemicals can cause mutations in the somatic cells of sexually reproducing organisms of all kinds. Much human cancer is probably caused by such means. So why should the germ line be immune from chemically caused mutation?

Two conflicting pieces of evidence make the question more interesting than it might at first sight seem. First, there is the surprising evidence, recently accumulated, that the children begotten of people exposed in 1945, at Hiroshima and Nagasaki, to radiation from the earliest nuclear weapons have been remarkably free of genetic aberrations caused by dominant mutations. Nobody knows what the explanation is. Are germ cells unexpectedly resistant to mutagenic influences, perhaps because they are dormant for most of their existence, or is the uterus a more efficient way of distinguishing between unwanted dominant mutations than has hitherto been supposed? That question will be more easily decided when more is known about the occurrence of recessive mutations among the descendants of the people exposed to the first nuclear explosions. Meanwhile, it remains a matter of public record (see *Nature* 154, 81; 1944) that the simple and naturally occurring chemical allyl *iso*-thiocyanate can cause inheritable genetic mutations in *Drosophila*. More recently, there have been reports that defoliants contaminated with dioxin and used in the recent war in south-east Asia have scattered inheritable genetic damage through the exposed population.

So it is natural that the US Environmental Protection Agency, required by law to anticipate (and to prevent) such occurrences, should have asked the US National Academy of Sciences to suggest how the potential of particular chemicals to cause germ-line mutations can be assessed without too much administrative trouble (and cost). The academy's Committee on Chemical Environmental Mutagens, itself an offshoot of the Board on Toxicology and Environmental Health Hazards, one of the dependants of the academy's Commission on Life Sciences, has now dutifully obliged*. The committee suggests that if the Environmental Protection Agency is bent on spotting inheritably mutagenic

chemicals before they cause damage, it had better rely on a combination of two diagnostic tests — some version of the Ames test (mutagenicity potential in *Salmonella*, for example) and an assay of potential for causing chromosome breaks in cultured mammalian cells, cells derived from Chinese hamster tissues for example. By all accounts, the agency is not sure how it should respond to this surprisingly pragmatic advice.

What the agency should do is to read the fine print in what is an exceedingly intelligent report. First, it must take the point that most chemicals that affect the life of organisms do so not in their own right but because of their metabolic products. Somatic mutagenic agents such as benzo(a)pyrene, for example, cause cancer only when they are converted into epoxides. Obviously, then, no simple combination of an Ames test and something else can be an unambiguous pointer to the inheritable damage that some chemical may do. And false negatives — assays in which a chemical appears innocuous but is not — will be more worrying (because they will not be immediately apparent) than false positives, still one of the major anxieties in the use of the Ames test of mutagenic potential in bacteria as a guide to carcinogenic somatic mutation in mammalian cells. Second, the Environmental Protection Agency must recognize that the telling import of the National Academy report is that it does not point to immediate legislation but, rather, to the need for some sensible programme of research. The goal should be to identify a few cases in which chemicals cause genetic alterations of the germ-line, and then to understand why they occur — and do not occur more often. It should be chastening for all that the paper by Auerbach and Robson, published nearly forty years ago (see above) is still the most often quoted.

Luckily for us all, the National Academy's report embodies a few important pointers to what might be done. The ideal, of course, would be that there should be such a full understanding of what happens metabolically to particular chemicals in mammalian cells (germ as well as somatic cells), and such a full catalogue of possible interactions with nuclear DNA, that the prediction of mutagenicity would be possible. For the time being, however, such an objective is entirely beyond the bounds of what can be attained. So the best hope is that some long-pocketed agency such as the

Environmental Protection Agency should support an empirical attempt to discover what there is in common between the chemicals — the list is short — known to be mutagens of the germ-line. The National Academy's committee has several useful suggestions as to how extra resources might be used in such an endeavour — the study of natural repair mechanisms or of the role of mobile genetic elements (transposons) in mutagenesis, for example. But the most urgent need is of some means for tackling a problem that usually goes unstated — that of estimating the true biological damage that may be done by chemical mutagens, allowing for mammalian fetuses prophylactically, so to speak, aborted and for biologically unimportant defects which, when inherited are equivalent to impotence.

None of these questions will be quickly answered, so that the Environmental Protection Agency is right to be saying (for the time being) that it has no specific programme of legislation up its sleeve. For as things are, the legislation on the toxicity of chemicals that already exists for practical purposes allows the use of chemicals that show up positive on a simple Ames test to be regulated. The consequence is that laws intended to control those chemicals that show up positive both on an Ames test and for their capacity to cause chromosome breaks in cultured mammalian cells are likely to be superfluous: culprit chemicals are almost certain to be caught by the net with the finer mesh. Indeed, the procedure that the National Academy recommends is either administratively irrelevant or it amounts to a plea for finding those mutagens of mammalian DNA that are not recognized by the Ames test.

The second course is entirely laudable, and should be followed as energetically as time and resources will allow. The danger, created simply by the agency's expression of interest in this question is the belief that to every problem there should be a matching piece of legislation. What trigger-happy lawyers and their paymasters must be made to understand is that the Environmental Protection Agency has asked an interesting question, that the National Academy of Sciences has given an intelligent answer, and that there is, for the time being, no way of telling what should be done to protect people against the hypothetical hazard of germ-line mutations by chemicals except by the support of basic research.

* *Identifying and Estimating the Genetic Impact of Chemical Mutagens* (National Academy Press, Washington AC, 1983).

Malaria prevention

How red blood cells and malaria parasites recognize each other

from F.E.G. Cox

IN this issue of *Nature*, Weatherall and his colleagues (see p.704) report the isolation of the proteins that appear to be involved in the recognition of red blood cells by the malaria parasite. The report brings forward the time when an antibody that blocks recognition of the red cell might be produced and raises hopes of preventing malaria by stopping this crucial step in the invasion process.

Infection with malaria begins when sporozoites are injected by a mosquito into a capillary of the host and are carried to the liver. There, asexual division produces thousands of merozoites which go on to invade red blood cells. Further asexual division then occurs, resulting in the formation of 8–16 or more merozoites which invade new cells and begin the period of red cell invasion and destruction characteristic of malaria.

The invasion of red cells by merozoites is a key stage in the infection and must take place through the recognition of specific red cell surface molecules¹. Of particular importance in the recognition of the red cells are their sialoglycoproteins, glyophorins A, B and C.

Human red blood cells have numerous well characterized polymorphisms and many lack particular blood-group antigens. This in turn makes it possible to obtain useful information on the receptors used by merozoites for red cell recognition and invasion. Cells of the type En(a⁻) are not susceptible to invasion by *Plasmodium falciparum*, the causative agent of malignant tertian malaria in man². Such cells lack glyophorin A, suggesting that this sialoglycoprotein is a merozoite receptor, as has indeed been shown to be the case³.

Glyophorin B has also been implicated as a possible receptor. Cells of the type S⁻U⁻ possess glyophorin A but lack glyophorin B and, as expected, are susceptible to invasion by merozoites of *P. falciparum*³. However, trypsin-treated S⁺s⁺U⁺ red cells lack glyophorin A but not B and are still susceptible to merozoite invasion, though at only 20–30 per cent of the level of cells with glyophorin A^{4,5}. The conclusion, therefore, is that glyophorin A is important for invasion and so is glyophorin B.

Glyophorins A and B are similar and relatively complex, so which of their con-

stituent molecules actually act as receptors? One candidate is *N*-acetylglucosamine, a sugar present at the N-terminal ends of glyophorins. The presence of *N*-acetyl-D-glucosamine inhibits red cell invasion^{4,6}, suggesting a competitive interaction with similar molecules on the red blood cells and implicating such molecules as the actual receptors.

In the experiments described in this issue of *Nature*, the parasite molecules that recognize the receptors have been isolated. Four metabolically labelled proteins of *P. falciparum* — one of 210K, two of 140K and one of 35K — bind to red cell sialoglycoproteins. One 140K protein and the 35K protein are specific to sugars. Further purification suggests that 140K, 70K and 35K proteins bind to *N*-acetylglucosamine. The three proteins seem to be related and probably represent a tetramer, a dimer and a monomer, so it is not possible to say exactly how many sugar-binding proteins are present on the

merozoite. It is possible to envisage a multi-step process of cell invasion in which the parasite binds to the sugar molecules first and then to more specific sites⁵ but in order to intervene in the normal process of invasion it would be necessary to block the primary binding sites. The paper suggests what these are and is a big step towards the day when a receptor-blocking antibody can be produced.

It would be nice to imagine that *N*-acetylglucosamine is the, or part of the, receptor but there are questions that must first be resolved. The experiments described in this issue are based on the observation that red cell invasion of merozoites is inhibited by *N*-acetylglucosamine and the conclusion that competition between this molecule and the receptors on the red cell is responsible. *N*-acetylglucosamine does, however, adversely affect malaria parasites directly and is toxic to the parasite within the red blood cell⁴. This complicates any interpretation of results implicating *N*-acetylglucosamine as a receptor but does not invalidate them. What is now needed is absolute proof. With facilities for isolating polypeptides and monoclonal antibodies becoming available this should soon be possible. □

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Oncogenic intelligence

Polymorphism or oncogene?

from Peter Newmark

THE latest issue of *Science* (18 February) contains a sobering thought for anyone still intoxicated by the reports (see *Nature* 300, 149; 1982) that a single base change distinguishes the *c-ras*^H transforming gene of the T24 bladder cancer cell line from its non-transforming counterpart in normal human DNA. The base change results in the substitution of valine for glycine in the p21 protein encoded by *c-ras*^H. Muschel *et al.* (*Science* 219, 853; 1983) now present preliminary evidence of an equivalent base change in the *c-ras*^H gene of a tumour from a patient with bladder cancer: the sobering fact is that his normal tissue contains an indistinguishable gene.

Before considering what that implies, the limits of the evidence need to be emphasized. Most importantly, Muschel *et al.* have not investigated whether the gene from either the tumour or normal tissue is able to transform NIH 3T3 cells, the biological assay that characterizes the T24 gene as a transforming gene (oncogene). Nor can it be assumed to be such while Muschel *et al.* only know, by restriction enzyme analysis, that glycine can no longer be coded for and do not know that the substituent is valine or some other amino acid that seems to confer transforming properties on p21. (Note that they claim that only

one of the two alleles of the gene is altered.)

Since substitution for glycine is found in both tumour and normal tissues (healthy bladder and white blood cells), what is now to be made of the claims that the substitution in T24 cells distinguished them from normal tissue and was a key factor in the conversion to malignancy?

The difference claimed between T24 cells and normal tissue may well be wrong for lack of availability of the ideal control — healthy tissue from the patient from whom the T24 cell line was derived. Unfortunately that patient is long since dead so that in one case placental tissue and in another case normal bladder epithelial cells were used to derive the control gene.

It now seems probable that the patient, like Muschel *et al.*'s, would have had the same gene in healthy as in tumour tissue. In other words, the *c-ras*^H gene is polymorphic, existing both in the form that encodes glycine and in forms that encode an alternative amino acid. The latter must be very infrequent in the population because Muschel *et al.* have not detected them in other bladder tumours (about 10) or in healthy individuals (about 35).

So what remains of the claim that the substitution is a key factor in malignancy? There is still no question (and there is

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6. Weiss, M.M. *et al.* *Exptl Parasit.* 51, 400 (1981).

corroborating evidence) that the substituted T24 gene is transforming whereas the unsubstituted version is not. But more clearly than ever, there is much more to malignancy than that — otherwise why did the patient of Muschel *et al.* have a tumour only in his bladder?

They adopt the reasonable fall-back position that the substituted gene, an infrequent polymorphism, produces a predisposition towards cancer, the

development of which in time and location requires other factors.

They go on to argue that the other factors must already have operated on NIH 3T3 cells (which are known to be far from normal). Addition of the substituted gene is the last straw — transforming an NIH 3T3 cell into a full-blown tumour cell. □

Peter Newmark is deputy editor of *Nature*.

Japanese intelligence

Now the great augmentation of the American IQ

from James R. Flynn

ON the basis of various samples used to standardize Wechsler IQ tests in Japan, Richard Lynn has argued that the mean IQ of Japanese children has risen by about 7 points over the last generation, putting them by 1975 some 11 points above their American counterparts¹. The same contentions were made a few years ago by Lester and Muriel Tarnopol citing some of Lynn's earlier work². Lynn has also argued that the advantage Japanese enjoy in terms of mean IQ is much enhanced at high IQ levels and this may have been a factor in their economic success compared with the US and other nations. In fact, all these contentions are seen to be suspect when viewed in the light of US IQ gains over the last generation and current white US norms. Comparison with white Americans is appropriate given Lynn's use of his data to make comparisons between Japan and nations largely populated by Europeans, such as the UK, Germany and Australia.

The evidence for American IQ gains has been published in detail elsewhere³ but it may be summarized as follows. In the US, great care is taken to ensure that the standardization samples of Wechsler tests are representative of the total population. Therefore, if the same group of subjects does better on an old test than a new one, the obvious explanation is that old norms are easier to exceed than more recent ones, which is to say that older standardization samples did not perform as well on IQ tests as more recent samples. Five studies in which American children took both the WISC (normed 1947–48) and the WISC-R (normed 1972) collectively revealed that they did 7.86 IQ points better on the earlier test, which suggests a rate of gain in IQ of 0.321 points per year over 24.5 years.

Seventeen studies using all combinations of Wechsler and Stanford–Binet tests during that period, 1948–1972, yielded a rate of 0.358 points per year or a total gain of 8.77 points.

The American gains are far more difficult to explain than the Japanese gains. As was pointed out by Anderson in a recent *News and Views* article, a Japanese gain of 7 points over a generation can easily be explained as almost every factor known to influence IQ can be brought into play⁴. Since 1930, Japan has experienced massive urbanization, a cultural revolution from feudal towards western attitudes, the decline of inbreeding and consanguineous marriages, huge advances in nutrition, life expectancy and education. But as Anderson points out, to find analogous massive changes for the US or Western Europe, one has to go back to the turn of the century. The magnitude of gain Anderson attempts to explain in the Japanese context is also present in the post-1948 American context. Whatever the explanation, the same sort of data cited to evidence Japanese gains — comparisons of WISC and WISC-R performance — shows Americans making IQ gains at a most impressive rate.

The best evidence Lynn has for his contention that the Japanese possess a mean IQ 11 points above Americans comes from the Japanese WISC-R standardization sample of 1975. All other evidence from Wechsler tests standardized in Japan either reveals no significant advantage over American whites (WAIS and WISC) or an advantage suspect because it is present only among pre-school children less than 7 years of age (WPPSI)⁵. Lynn scored the Japanese standardization sample against the norms set by the American WISC-R sample of 1972 and derived an overall mean for ages 6–16 of 110.7, as compared with the American mean of 100. However, as we shall see, much of this apparent advantage is open to serious challenge.

First, Lynn used performance on only five subtests of the WISC-R to compute his mean, setting aside two verbal subtests —

Digit Span and Arithmetic. One problem was altered on the Arithmetic test but that was also true of Block Design which Lynn includes. Using performance on all seven subtests that were essentially unaltered in going from the US to Japan⁶, the Japanese mean falls by just over one point. Second, white Americans scored 2.26 points above the norms set by all Americans in 1972, because the all-American score includes data from low-scoring racial minorities⁷. If Japanese are scored against only white Americans, then another two points can be deducted. Third, Americans were gaining about one IQ point every three years during this period; since the American sample was tested in 1972 and the Japanese in 1975, another point must be deducted if the latter are to be scored against current rather than obsolete norms. The table details how the mean IQ of the Japanese WISC-R standardization sample must be lowered from 110.7 to 106.6 to conform to current white American norms (columns I–IV).

Japanese WISC-R standardization sample: scored against American norms

Year of birth	I	II	III	IV	Age when tested
1959	106	108	106	105	16
1960	109	109	107	106	15
1961	111	111	109	108	14
1962	111	111	109	108	13
1963	112	112	110	109	12
1964	111	111	109	108	11
1965	111	109	107	106	10
1966	112	109	107	106	9
1967	112	109	107	106	8
1968	111	108	106	105	7
1969	112	109	107	106	6
All ages:	110.7	109.6	107.6	106.6	

The four means were scored as follows:

- I. Five subtests, norms all Americans, 1972
- II. Seven subtests, norms all Americans, 1972
- III. Seven subtests, norms white Americans, 1972
- IV. Seven subtests, norms white Americans, 1975

When Lynn argues that the Japanese advantage at the mean is enhanced at high IQ levels, he makes the mistake of overlooking the fact that, as explained in a *Matters Arising* published in this issue of *Nature*⁸ (p.738), the Japanese standard deviation is less than the white American one, 12.81 (ref.8) as compared with 14.14 (ref.9) on the performance scale. At an IQ of 130 and above, where we find 2.3 per cent of white Americans, there would be 4.5 per cent Japanese rather than the 10 per cent claimed. Indeed, there would be the same percentage of Americans over 125 as Japanese over 130, hardly a matter of national concern. As for the notion that IQ differences of this magnitude, say 5–7 points, have influenced economic history since World War II, recall the fact that white Americans have gained 8 or 9 points during that time. It seems unlikely that this sort of IQ advantage really means that Americans today are so much brighter than their parents as to be able to overwhelm them in economic competition. □

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2. Tarnopol, L. & Tarnopol, M. *Focus Learning Pbs Math.* 2, 34 (1980).

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Fish vision

... all the better
for catching
Daphnia with

from J. N. Lythgoe

THE most important factor that limits the fineness of detail that a fish can see is the size and packing of the cones in its retina. A new study that has recently appeared in *Science* (218, 1240; 1983) shows the practical effect of this for the bluegill sunfish — the larger it grows the smaller the prey it can see and catch.

The techniques used by Nelson Hairston, Kao Li and Stephen Easter in their study of the bluegill sunfish (*Lepomis macrochirus*) were quite simple. Water fleas (*Daphnia*) of known size were introduced into a shallow tank containing several fish whose feeding manoeuvres could be monitored with a vertically mounted video camera. On sighting a *Daphnia* a fish would turn, swim towards it and snap it up. The distance between the point of turning and the snap was taken as the reaction distance. It was then possible to calculate the visual angle subtended by the *Daphnia* at about the time that the bluegill recognized it was good to eat.

It was found that the visual angle subtended by the *Daphnia* at the time of recognition varied from a mean of 27.8 minutes of arc for fish 3.7 cm long to 14.2 minutes of arc for larger fish 5.8 cm long.

The observation tank was brightly lit and it is unlikely that vision was limited by insufficient light, or indeed that the rods, which are the receptors for dim light vision, were involved at all. In these conditions the detail that an eye can resolve is limited by two fundamental factors. The first is interference to light as it passes through the pupil aperture, causing blurring of the image on the retina in a mathematically definable way. The second factor is the spacing between the centres of the retinal receptors (in this case the cones) which limits the detail with which the image of the visual scene on the retina can be sampled.

For the bluegill sunfish it is the spacing of the cones rather than interference at the pupil aperture that limits the ability to resolve detail. The twofold improvement in visual acuity as the fish grows is due to an increase in the number of cones that sample the image. The spacing of the cones themselves remains quite constant, but the area of retina, and hence the number of cones available to sample the larger image, increases as the eye grows larger.

The study reinforces the belief that big eyes are best. This is not because small eyes are less sensitive, as the brightness of the retinal image from an ordinary visual scene depends on the ratio of pupil aperture to focal length (the *f* number used by photo-



A bluegill sunfish colony: bigger fish have bigger eyes and can see smaller prey. (Drawing from Dominey, W. J. *Nature* 290, 586; 1981.)

graphers). Provided this ratio remains constant, as it probably does in these eyes, the brightness of the retinal image is the same whatever the size of eye. Where there does seem to be a limit is in the cross-sectional area and packing density of the cones themselves. Thus the retinal 'grain' cannot be reduced beyond a certain point and a small retinal image cannot yield so much information as a large one.

One limit to the size of the cones themselves relate to their properties as a light guide. The outer segments, which contain the light-sensitive visual pigment, have a lighter refractive index than the surrounding cytoplasm and hence act as tiny light guides. In the sunfish the outer

segment of each cone is about 2,000 nm across. If their diameter were much smaller than this, the light-guiding property of the outer segments would probably be reduced, leading to light leaking across from one cone to its neighbours. Thus the capacity of small fishes, and indeed that of any small animal, to see fine detail is likely to be limited by the size of the eye itself and as the authors of the *Science* paper point out, this will provide a selective advantage for the continuous retinal growth noted in many fish. □

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Non-linear systems

Coherent excitation in biology

from F. Keilmann and D. B. Kell

SUPPLY of energy to a material system may lead to the excitation of a coherent mode in which the motion of many of the particles that compose the system becomes highly correlated. Such coherent excitation is seen in masers and lasers, for example, and involves a nonlinear change in certain properties of the material. Some fifteen years ago H. Fröhlich (*Int. J. Quantum Chem.* 2, 641; 1968 and *Adv. Electronics Electron Phys.* 53, 85; 1981) conjectured that coherent excitation of electrical oscillations might play an important part in biological as well as physical systems. Recently, physicists and biologists met in Germany to discuss progress in assessing the nature and existence of such coherent excitations in biological systems. The general conclusions of the meeting* were that coherent excitation had now been shown to be an intimate feature of biological activity; further progress would rely on detailed studies of well characterized material.

In his early work, Fröhlich carried out a theoretical study of the electrical oscillations of sections of biological membranes and showed that a supply of metabolic energy above a critical rate could

lead to coherent electrical oscillations. Such excitations are not, however, restricted to membranes and it is possible for interactions to occur between systems of equal frequency at a distance at which ordinary chemical interaction is absent and electrostatic interaction is screened. The possibility of coordinated activity of large regions thus appears. Estimates of the frequency of coherent oscillations of membranes gave values in the 10^{11} Hz range — the same as that of electromagnetic millimetre waves. If biological systems do in fact make internal use of such excitations then great sensitivity to external radiation at the relevant frequencies should be expected. Work reported at the conference suggests such sensitivity exists.

A remarkable 44 per cent increase in the fertility of *Drosophila melanogaster* was reported (G. Nimtz, University of Köln) to occur in the first generation after irradiation of pupae for 5 days with microwaves (40 GHz) at an intensity of only $10 \mu\text{W cm}^{-2}$. In a study of puffing patterns in the giant chromosomes of *Acrisotopus lucidus* (F. Kremer, Max-Planck-Institut, Stuttgart), a fivefold increase in the number of condensations of Balbiani rings ($\alpha < 0.5$ per cent) was observed after only 2 h of low-intensity irradiation at 64–69 GHz. Reproducible

*The international symposium on 'Coherent Excitations in Biological Systems', held at Bad Neuenahr on 29 November–1 December 1982, was sponsored by IBM Germany; chairman H. Fröhlich. The proceedings are to be published by Springer Verlag.

changes of about 10 per cent in the average growth rate of yeast cells *Saccharomyces cerevisiae* (W. Grundler, Gesellschaft für Strahlen- und Umweltforschung, Neuherberg), were observed at several frequencies near 42 GHz. Use of fine-scaled frequency tuning showed the response to be highly resonant, such that detuning by only 1 part in 10^4 obliterated the effect. The sharpness of frequency response together with a step-like dependence on the microwave intensity is in agreement with theoretical requirements.

When human red blood cells are dispersed in plasma they appear to carry out brownian movement until they attach to each other, forming rouleaux which, under the microscope, have the appearance of stacks of coins. In extensive microscopic investigations, S. Rowlands (University of Calgary) found that the erythrocytes appear to rush forwards towards each other once they have approached to within about $4\ \mu\text{m}$. Analysis of the movement in terms of the Smoluchowski theory permits a quantitative evaluation of the interaction. If the attraction arose from coherent membrane oscillation then the measured attraction satisfies the requirements of the theory; it disappears when the membrane potential is removed by a change of the plasma pH, or when they are depleted of their metabolic energy. The attraction reappears when both are restored.

What kind of biological structures might be responsible for these remarkable findings? Current ideas on the ultrastructure of various cells (J. Clegg, University of Miami) lay emphasis on the microtubular lattice and its attendant 'bound' water as a possible vehicle for cytoplasmic organization in general and for the transfer of coherent excitation in particular. Mouse fibroblasts (L cells) suspended in 0.3 M sorbitol contained only $0.5\ \text{g H}_2\text{O}$ per g dry mass, and were virtually in the solid state, yet metabolized in a fashion indistinguishable from that of 'normal' fibroblasts. Evidently a cytoplasm exhibiting a bulk aqueous milieu is not a feature crucial to cellular function.

It is now widely recognized that the membranous systems of electron transport phosphorylation effect free-energy transfer by a bioelectrical mechanism, in that spatially separate protein complexes have been shown to act as more-or-less reversible proton pumps. Various studies, however, led to the conclusion (D. Kell, University College of Wales) that the free-energy transfer was effected between specific individual complexes, so that traditional stochastic ensemble models must be proscribed. A coherent excitation is thus a particularly attractive possibility for explaining the properties of this process. $\square$

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Chandler wobble

Simulation shows wobble period neither multiple nor variable

from F.A. Dahlen

THE Chandler wobble, a 14-month free nutation of the Earth's principal axis of inertia about its rotation axis, has been continuously monitored for nearly a century. In spite of that, agreement about the fundamental nature of the observed motion is still lacking. Numerous workers, beginning with Chandler himself¹, have suggested that its period is either multiple or variable in time. The alternative view, which is preferred by most geophysicists and astronomers, is that the Chandler wobble is a single damped free oscillation of the Earth, maintained by some irregular excitation process. An article by Okubo in the December 1982 issue of *The Geophysical Journal*² should suffice to convince all but the most steadfast sceptics that the conventional view is correct.

Okubo has performed a straightforward numerical experiment. He generated an artificial Chandler wobble time series consisting of white noise passed through a single sharp resonance peak, and then subjected it to exactly the same analysis procedures used by those who have argued for a more complicated deterministic data structure. Colombo and Shapiro³ and Gaposchkin⁴, for example, have proposed that the Chandler wobble actually consists of two independent modes of oscillation separated in period by about 10 days. At first sight, such a conclusion seems strongly supported by the distinct 40–50-year beating visible in the twentieth century polar motion data after removal of the annual wobble; the beating leads in turn to a double Chandler peak in the polar motion periodogram. Okubo shows, however, that both these features are the expected consequences of passing a random input signal through a high- Q resonance peak. The beating is a result of interference between the two flanks of the peak, which gives rise to irregular amplitude variations on a time scale of order QT , where T is the resonant period. The beat period that emerges is consistent with the measured Chandler wobble with Q in the range 50–100. The double peak can be attributed to the well known statistical fluctuations inherent in periodogram spectral analyses of stochastic processes. The presence of a single damped mode is also corroborated by nineteenth century data collected and analysed by Yatskiv, Korsun and Rykhlova⁵. Their series from 1846 to the present shows irregular amplitude variations, which come to resemble regular harmonic beating during the twentieth century but not before.

Others, including Melchior⁶, Sekiguchi⁷ and Carter⁸, have postulated that the

Chandler period varies with time as a result of some underlying change in the properties or response of the Earth. These claims are generally based on a form of running spectral analysis, which shows evidence for a variation in instantaneous frequency, inversely correlated with amplitude, when applied to the Chandler data. Okubo examines several such methods and shows that in all cases virtually identical results are obtained when they are applied to his synthetic series. He concludes that "there is no positive evidence for the variable period model". The occurrence of instantaneous frequency variations of 1–2 per cent in the Chandler data is just another natural consequence of the random excitation of a damped harmonic oscillator with Q in the range 50–100.

Either a multiple or a variable period would be difficult to reconcile with existing theory, which predicts with some assurance that the Chandler wobble is a single damped free oscillation of the Earth well isolated in the eigenfrequency spectrum. Smith and Dahlen⁹ have recently shown that both its period and damping can be accounted for quantitatively by allowing for a slight frequency dependence of the shear dissipation in the mantle, of the form $Q_\mu \sim \omega^\alpha$ where ω is frequency and $\alpha = 0.1$ – 0.2 . This substantiates a conclusion about the Earth's anelastic rheology first drawn by Jeffreys¹⁰ in 1958.

The outstanding problem in the study of the Chandler wobble is, and has been for some time, the source of its excitation. Okubo's work confirms the generally accepted conclusion that the excitation has the statistical properties of quasi-stationary broadband noise. Earthquakes and atmospheric motions have both been discounted, as neither can account for the observed level of excitation. Hopefully, some clues will soon be forthcoming from the increasingly refined techniques of spatial geodesy, very large baseline interferometry and satellite ranging. $\square$

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Atherosclerosis and hyperlipidaemia

Genetic polymorphism and plasma lipoproteins

from Gilbert Thompson and Anne Soutar

ATTEMPTS to understand genetic disorders of lipoprotein metabolism, such as familial hypercholesterolaemia¹ and type III hyperlipoproteinaemia^{2,3}, have so far been made by looking for abnormalities of protein structure and function in individuals with clearly defined diseases. Now, advances in recombinant DNA technology are making it possible to search for a defective gene even when no specific protein abnormality has been identified. There was never any doubt that hyperlipidaemia and atherosclerosis would provide a fertile field of study for such genetic analysis and two papers — one appearing in this issue of *Nature*⁴ (p.718) — show that the bandwagon has just begun to roll.

cDNA probes for the human gene for apolipoprotein A-I (apoA-I), the major protein of high-density lipoprotein (HDL), have been isolated^{5,6} and are now being used for restriction enzyme analysis of human chromosomal DNA. In the work reported in this issue, Karathanasis and colleagues describe abnormalities closely linked to the structural gene for apoA-I in a family exhibiting HDL deficiency and premature coronary heart disease. The authors suggest that the genetic defect impairs synthesis of apoA-I and thus causes HDL deficiency and atherosclerosis.

Simultaneously, Rees *et al.*⁷ have reported that almost 50 per cent of a group of patients with hypertriglyceridaemia have, in the region flanking the 3' end of the apoA-I gene, a genetic polymorphism that is present in only 5 per cent of normal subjects. While it is by no means certain at this stage that the polymorphism is in any way responsible for the hypertriglyceridaemia, the study clearly demonstrates that a fairly diverse group of hyperlipidaemic patients do share an uncommon genetic marker.

The two sisters studied by Karathanasis *et al.* exhibited a unique combination of clinical and biochemical abnormalities, including deposition of cholesterol ester in skin, tendons, cornea and coronary arteries. The only factor linked to an increased risk of coronary heart disease was a marked reduction in HDL cholesterol, reflecting a deficiency of apoA-I. Restriction enzyme analysis using a cDNA probe for the apoA-I gene revealed an abnormality that was compatible with either a large deletion or an insertion in a region of the DNA that could be involved in regulation and transcription. First-degree relatives who were found to be heterozygous for the abnormal gene had intermediate HDL levels but were asymptomatic. The precise effect of the abnormal gene on the metabolism of HDL,

the significance of the accompanying deficiency of apoC-III, an apoprotein of both very low-density lipoproteins (VLDL) and HDL, and the relevance of these anomalies to atherosclerosis remain to be established.

But what mechanism underlies the epidemiological evidence that a low level of HDL in plasma is an independent risk factor for premature coronary heart disease? It may well be that the lipoprotein acts as a 'shuttle' for the transport of cholesterol from peripheral tissues to the liver, the only site where cholesterol can be catabolized and excreted in the bile⁸. The processes involved can all be demonstrated *in vitro*, although there is little real evidence that the whole scheme operates *in vivo* to prevent atherosclerosis.

To complicate matters, the origins of plasma HDL are diverse and it is very heterogeneous. Much of the polar lipid and the apoprotein associated with HDL comes from redundant surface material released during the intravascular hydrolysis of the triglyceride-rich lipoproteins, VLDL and chylomicrons. Generation of plasma HDL thus partly depends on the turnover of these triglyceride-rich lipoproteins — an observation that explains the well known inverse correlation between HDL cholesterol and VLDL triglyceride levels in plasma. Direct secretion of 'nascent' HDL (essentially HDL particles with a core devoid of cholesteryl esters) can, however, be demonstrated both from the liver⁹ and small intestine¹⁰ in experimental animals. Many factors may thus be involved in determining the input of newly synthesized HDL into the circulation, any one of which could be more closely linked to protection against atherosclerosis than is the steady-state concentration of HDL cholesterol in plasma.

The findings of Rees *et al.*⁷ demonstrate an alternative approach to that of Karathanasis *et al.*⁴. Using a different cDNA probe and restriction enzyme, they found a polymorphism in the 3'-flanking region of the apoA-I gene in roughly 5 per cent of a random population but in more than 40 per cent of a group of hypertriglyceridaemic patients exhibiting a lipoprotein phenotype of the type IV (excess VLDL) or type V (excess chylomicrons and VLDL) varieties. Two of these patients appeared to be homozygous for this particular form of apoA-I genetic polymorphism. The authors suggest that the abnormality might be responsible for the hypertriglyceridaemia but they provide no data to show that apoA-I or HDL levels were abnormal in their patients, nor do they state whether hypertriglyceridaemia was more marked in the two individuals

whom they identified as being homozygous for the genetic defect.

Hypercholesterolaemia due to increased levels of LDL in plasma has a causal role in atherosclerosis, as best exemplified by familial hypercholesterolaemia where a two- to fourfold increase in serum cholesterol throughout life increases the risk of premature coronary heart disease by up to 10-fold¹¹. However, the atherogenic potential of hypertriglyceridaemia resulting from an increase in VLDL is a subject of continuing controversy¹². Some believe that the increased risk of heart disease in hypertriglyceridaemic subjects is simply a reflection of the accompanying decrease in HDL. Furthermore, as discussed earlier, it remains to be proved that the undoubted association between reduced levels of HDL cholesterol and increased risk of heart disease represents a cause and effect relationship. The apparent lack of premature atherosclerosis in at least two familial disorders characterized by deficiency of HDL — apoA-I_{Milano} (ref.13) and fish-eye disease¹⁴ — argues against such a relationship.

But despite these reservations, delineation of the role of genetic factors in determining the concentration of VLDL and HDL in plasma will undoubtedly provide a better understanding of variations in serum lipids between individuals and may also help explain their differing susceptibility to atherosclerosis. Although the studies of Karathanasis *et al.* and Rees *et al.* are only preliminary, they do represent the beginnings of what could prove to be a very fruitful line of enquiry. □

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Control of gene expression

Cyclic AMP and development in the slime mould

from Robert Kay

THE slime mould *Dictyostelium discoideum* has proved particularly attractive to molecular biologists attempting to understand how the sequential expression of genes during development is controlled. *Dictyostelium* passes through a number of definite developmental stages — an amorphous mass of cells, formed by the aggregation of *Dictyostelium* amoebae, organizes itself into a cylindrical slug and finally into a fruit composed of a mass of spores supported by a stalk — and the changes in morphology are rigidly coupled to changes in gene expression¹. Especially valuable to researchers is that deliberate disaggregation of the slug into isolated cells halts and eventually reverses differentiation. Progress has been particularly rapid in recent months²⁻⁵ and here I shall sum up our current understanding of developmental gene expression in *Dictyostelium*.

It is easiest to start with the extracellular signals. Work with isolated cells shows that cyclic AMP induces both stalk and spore cell differentiation, and the choice between these developmental pathways probably depends on two endogenous morphogens: DIF, favouring stalk formation, and ammonia, favouring spore formation⁶. At the molecular level, most is known about cyclic AMP, which induces the accumulation of whole blocks of developmental proteins, partially at least by controlling their mRNA levels. It does this both by controlling transcription⁷ and, as has just been reported in *Nature*, by specifically stabilizing developmental mRNAs². In undisturbed slug cells, both developmental and housekeeping mRNAs turn over with a half life of several hours but, if disaggregated, many developmental mRNAs (detected by hybridization to their cloned counterparts) are then degraded with a half life of about 30 minutes whilst the housekeeping messengers are left unscathed. Cyclic AMP added to the isolated cells reverses this effect.

How the cell distinguishes housekeeping from developmental mRNAs is a mystery made all the more tantalizing by the fact that many developmental messengers are marked by a repetitive sequence⁸. Disaggregation also causes the disappearance from the cell of various developmental enzymes and even of an entire organelle (the prespore vacuole) — once again all this can be prevented by cyclic AMP. It is thus likely that cyclic AMP stabilizes developmental proteins, just as it does mRNAs. There is also a possibility that control is exerted at the nuclear RNA processing stage. In the developing cell there are three kinetically distinguishable processing routes: a rapid one for housekeeping functions, a slower one for some developmental messengers and a very slow one, involving extensive RNA degradation, for developmental messengers having a repetitive element⁹. It will be very interesting to see whether cyclic AMP has any effect on these processing routes.

The picture that emerges is of cyclic AMP inducing cell differentiation using chains of command that radiate out to many control points. Disaggregation of the slug has clearly proved a useful tool for revealing these effects of cyclic AMP — but why does it work? The only reasonable inference is that disaggregation disrupts a cyclic AMP signalling system in the slug, resulting in cyclic AMP depletion¹⁰ and hence responsiveness of the isolated cells to exogenous cyclic AMP. Such a signalling system in the slug has always been expected by those who believed that the cyclic AMP

system responsible for the chemotactic aggregation of the amoebae would be too useful to lose in later life.

Recently attention has focused on how a cell chooses between stalk and spore differentiation. Conventional wisdom has this decision made, albeit reversibly, as the slug forms. Things may not be quite so simple, however. Two groups have recently cloned mRNAs specific to prestalk and prespore cells^{3,4}. Although many of the mRNAs start accumulating at the expected time some prestalk-specific mRNAs appear even before aggregation. This could mean that prestalk cells differentiate much earlier than previously thought but it is more likely that these mRNAs first accumulate in all cells and are later lost only by the prespores. This certainly seems to be the case for the major actin mRNA of vegetative cells which later in development is seen only in prestalk cells¹¹. The expression of both prestalk- and prespore-specific genes is stimulated by cyclic AMP, supporting the idea that cyclic AMP is not of itself pathway-specific. One is left wondering instead how the cyclic AMP mechanism is 'targeted' by DIF and ammonia to its appropriate genes.

Finally, what new attraction is most likely to bring the present-day molecular biologist to *Dictyostelium*? The organism is unique for the ease with which developmental mutants can be isolated — development is facultative so no function, however vital, is immune from the geneticist. Using transformation¹² and perhaps the newly discovered endogenous plasmid⁵ we can therefore expect to isolate regulatory genes by the direct complementation of existing mutants. With this additional resource developmental gene expression in *Dictyostelium* may become an open, though very complicated, book. □

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The river Cuango at Yacca, the furthest point reached by the Capello-Ivens expedition to trace the source of the Cuango, a tributary of the Congo. From *Nature* **27**, 393, 22 February 1983.

Microtubules in action

Multiple mechanisms of mitosis?

from Stephen King

DURING mitosis chromosomes are associated with a framework of microtubules, the varying arrangements of which form a plethora of different spindle designs. It is a matter of some controversy as to whether these differences in spatial organization are reflected in a comparable mechanistic diversity or whether the popular idea of a 'unified mitotic mechanism' (one applicable to all eukaryotic cells) is, in fact, correct. Recently, data have been obtained from several different laboratories which suggest that microtubules of the mitotic apparatus may be involved in several distinct motile reactions.

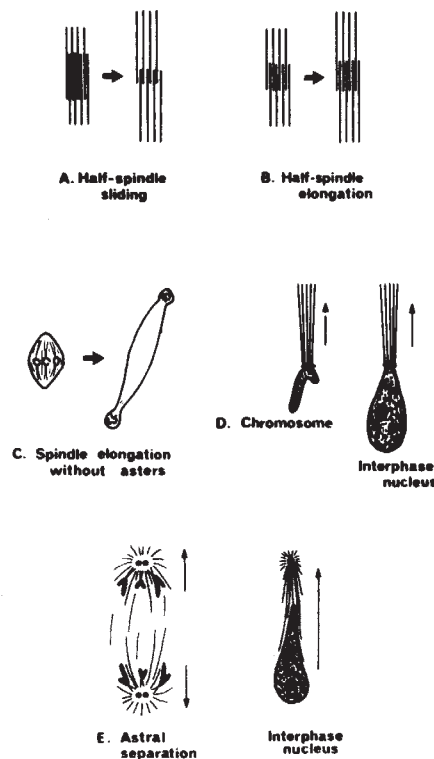
One particularly intriguing result has been obtained from the examination of mitosis in endosperm of the plant *Haemanthus*¹ during treatment with taxol (a drug that stabilizes microtubules and also promotes their assembly²). In these experiments, the anaphase movement of chromosomes was desynchronized and for periods of up to 10 min the direction of motion was reversed before polar migration resumed. As shown by immunogold staining, microtubule elongation occurred in the direction of chromosome movement. The data thus suggest that taxol-induced elongation produces a pushing force *in vivo*. However, whether microtubules do, in fact, exert a significant 'push' in the absence of taxol still remains open to question. But bearing in mind this caveat these experiments do provide evidence for chromosome movement as a result of microtubule growth.

The reversible anti-microtubule drug nocadazole has been used to arrest chromosome movement in early anaphase mammalian (PtK₁) cells³. Almost complete disassembly of non-kinetochore microtubules (those not directly attached to the chromosomes) results but following release from drug-induced arrest, spindle elongation (anaphase B) and chromosome-to-pole movements (anaphase A) both restart without the reappearance of the disrupted microtubules. This is a highly interesting result as it strongly suggests that anaphase B does not require interzonal microtubules and also that anaphase A motions are not based on an interaction between kinetochore and non-kinetochore microtubules.

Other studies on this cell line have also suggested that interzonal microtubules do not generate the motive force required for anaphase B (ref.4). When microtubule continuity between the two half-spindles was disrupted by pushing the dorsal and ventral membranes of the cell together, the rate of anaphase B essentially doubled.

Although reservations have been raised concerning this experiment it is interesting that the results obtained are compatible with those from nocadazole-treated cells.

Both sets of results are apparently at odds with those obtained using permeabilized models of PtK₁ cells⁵. In this system, anaphase B movements continued in conditions similar to those that bring about the sliding disintegration of flagella axonemes, suggesting the presence of a dynein-like Mg²⁺-ATPase in the spindle⁶. Further evidence for the active involvement of spindle microtubules in



Some of the mechanisms suggested to be involved in anaphase movements. These models are supported by evidence obtained from many different organisms. Thus it appears improbable that a single force-generating mechanism could be responsible for the anaphase separation of chromosomes in all eukaryotes.

anaphase B has been obtained following microbeam experiments on diatoms⁷ where interdigitating microtubules slide apart to allow for the observed elongation.

In another series of microbeam experiments, however, a laser was used to disrupt the spindle of the fungus *Fusarium solani* and brought about a threefold increase in the velocity of anaphase B (ref.8). This led to the hypothesis that the

microtubules function as a governor regulating some unidentified force-generating component (perhaps the astral microtubules). A similar suggestion has also been advanced following an analysis of anaphase B in *Saccharomyces cerevisiae*. Here, the central spindle is composed solely of continuous microtubules and as spindle length increases from 1 to 4 μ m the number of microtubules rapidly decreases from 10 to 1. This single microtubule spindle then continues to elongate, reaching a final length of 8 μ m (ref.9). Correlation of the decrease in microtubule number with the timing and velocity of anaphase B (ref.10) has shown that at the final transition from two microtubules to one there is an 11-fold increase in the rate of spindle elongation — strong evidence of a passive regulatory role for the continuous microtubules.

Recently it has been hypothesized that in diatoms, chromosome movement is achieved by a motile kinetochore (the chromosome-microtubule attachment site) which utilizes the spindle microtubules as a vectorial framework¹¹. Results of experiments using metabolic inhibitors to deplete intracellular ATP levels¹² suggest that it is the establishment of the metaphase configuration of chromosomes that requires ATP. Thus metaphase is proposed to be a 'high-energy' state and anaphase A motions might be achieved by the release of this tension (after chromosome splitting) via the kinetochore. Whether such a hypothesis may be applied to other system is uncertain as diatoms have unusual chromosome and spindle arrangements, but the possibility should not be ignored.

In conclusion, the proposed ubiquity of models based on interactive phenomena is being increasingly challenged. Indeed, the concept of phylogenetic universality¹³ has proved to be the most difficult hurdle for any model of mitosis. But it should be remembered that many current hypotheses already invoke two different roles for the spindle microtubules — sliding and assembly/disassembly⁵ — and there is no *a priori* reason why other mechanisms using microtubules as a vectorial framework^{11,12} or as a regulatory system^{8,10} might not also have evolved. Indeed, as more functionally significant information is gathered, the possibility of multiple mechanisms of mitosis becomes increasingly attractive. □

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The centre of the Galaxy

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The centre of a galaxy attracts attention, because of its unique location and often intense activity. It also poses two outstanding puzzles. The first concerns the source of the enormous power in the form of radiation and high velocity gas pouring out of the centres of some galaxies. Second is how galaxies formed at all after the randomizing blast of the big bang, and whether some unique massive objects at their centres may have played a part. The centre of our own Galaxy seems rather tame, in the past having relatively minor activity by comparison with some of the more spectacular galaxies. However, extraordinary conditions do exist, and there are suggestions that it has produced high velocity gas or transferred large amounts of momentum to material circulating around it. Furthermore, because it is our closest galactic centre, we study it in the most detail.

DURING the past few years, radio and IR astronomers have assembled a large body of information about the inner few light years of the Galaxy. X-ray and γ -ray observations have also been made from balloons and spacecraft, and unusual sources have been detected in the general direction of the galactic centre. These observations and their interpretation are the principal subject of this review. Among other things, they show that within the inner few parsecs (hereafter denoted 'the nucleus') lies the densest concentration of stars in the Galaxy as well as a quantity of ionized gas and warm dust. Most of this nuclear gas and dust is clumped into a small number of individual clouds. Little information is as yet available concerning the motions of individual stars, but the clouds are known to have high velocities (approaching $\pm 300 \text{ km s}^{-1}$), and to be expanding rapidly. Exotic explanations, involving massive black holes, as well as more conventional models have been proposed to account for the observations. Neither the origin of these velocities nor the source of the ionizing and dust-heating radiation in the nucleus has been definitely established, although the extent of evidence now available narrows them down to a very few possibilities. Detection of electron-positron annihilation radiation and a peculiar radio 'point' source very near the galactic centre add to the belief that the nucleus may contain some unusual object such as a black hole. In addition, clouds apparently moving away from the galactic centre and anomalies in one of the Galaxy's spiral arms suggest violent activity in the past¹. Although no unique interpretation has emerged, we now have a wide variety of observations of the galactic nucleus through the obscuring dust and some very solid understanding of its nature, but also puzzling glimpses of poorly understood phenomena which challenge both experimental technique and theoretical interpretation. These provide a view which is much more detailed than that available for any of the other, more distant, galactic nuclei and put severe restrictions on the range of possible models for this unusual region.

Stars

A star cluster at the galactic nucleus is to be expected from what we know of other galaxies, but it was not until 1968 that Becklin and Neugebauer² were able to make the first detection of such a cluster as a result of their early IR work. They detected a bright area of $2.2 \mu\text{m}$ radiation from the direction of Sgr A with an angular distribution somewhat similar to that of the radio emission from ionized gas. The near-IR colour of the radiation matched that expected from a cluster of stars obscured by an amount of dust which could reasonably exist in the galactic plane along the line of sight to the centre. Although only 10^{-10} of the visible starlight is transmitted through this dust, nearly 10% of the $2.2 \mu\text{m}$ radiation from the galactic centre penetrates,

allowing detection at the Earth. By assuming that the relative numbers of various types of stars is like that in the optically observable nucleus of M31, it was estimated that $\sim 10^7$ solar masses ($M_\odot$) of stars are found within 3 pc of the centre³. The light emitted per unit mass by a cluster of stars depends strongly on the types of stars present, so that an accurate determination of the mass of the nuclear star cluster directly from its $2.2 \mu\text{m}$ luminosity is not possible. Nevertheless, it is clear that the density of stars in the galactic centre exceeds that in the solar vicinity by a factor of at least 10^7 .

The angular distribution of the near IR radiation has been extensively studied. A $2.2 \mu\text{m}$ 'picture' of the central 1° of the Galaxy⁴ is shown in Fig. 1. The concentration of stars in the galactic plane, which runs diagonally across the picture, and the additional concentration towards the centre are apparent. A more detailed map of the $2.2\text{-}\mu\text{m}$ emission from the central arc min (ref. 5) of Fig. 1 is shown in Fig. 2. The appearance of this region is dominated by individual red giants near the galactic centre. Extended emission is also seen which is approximately centred on the source in Fig. 2 labelled 16. Recent imaging of the central region near $1 \mu\text{m}$ by arrays of detectors shows two additional stellar-like sources⁶⁻⁸, which are too highly obscured to allow detection in the visible region and too faint at $2.2 \mu\text{m}$ to be prominent at that wavelength. These may be simply normal stars lying in front of the nucleus, or they may possibly be something more significant.

Spectra of several of the discrete near-IR sources show absorption bands of CO, indicating they are red giants—stars which are probably more massive than the Sun and near the ends of their most luminous phase. Only a few of these sources have been observed with high enough spectral resolution to measure the Doppler shifts of their spectra. Their velocities along our line of sight vary from $-170 \pm 50 \text{ km s}^{-1}$ to $70 \pm 75 \text{ km s}^{-1}$ (ref. 9), comparable with the observed gas velocities, though not yet very accurately determined. If many stellar velocities could be measured, the mass density, which determines the gravitational field in which they orbit, could be determined. However, this is not yet possible with the sensitivity of equipment available.

A cluster of stars of similar mass should, if dense enough and old enough for near collisions to produce equipartition of kinetic energy, have a density distribution proportional to r^{-2} , where r is the distance to the centre. However, for very small values of r , the density becomes approximately constant rather than diverging. Such a distribution makes the total number of stars (or hence the total luminosity) inside a given radius proportional to r . This was in fact the luminosity distribution found by Becklin and Neugebauer, within uncertainties due to varying obscuration over the region.

Dust

At wavelengths λ beyond about $4\ \mu\text{m}$, emission from warm interstellar dust is much more prominent than that from stars, which decreases as λ^{-2} . The radiation near $10\ \mu\text{m}$ (ref. 10), shown in Fig. 3, is predominantly due to dust heated to temperatures of a few hundred Kelvin by the intense radiation fields in the nucleus. Dust, small ($\approx 0.1\ \mu\text{m}$) solid particles made largely from the heavier atoms such as C, N, O and Si, normally represents about 1% of the interstellar matter, which is itself a small fraction of the mass of stars in this region. However, a significant fraction of the stellar radiation is absorbed and reradiated by dust. Some of the warm dust can be seen from Fig. 3 to be concentrated in patches of typical size $\sim 5\ \text{arc s}$ or $\sim 0.25\ \text{pc}$. These patches also contain ionized gas.

Emission at still longer IR wavelengths, $\sim 100\ \mu\text{m}$, has been studied during the past few years with instruments in balloons or in NASA's Kuiper Observatory, a high-flying airplane containing a 36 inch telescope and operating above most of the atmospheric water vapour. The bulk of the luminosity observed from the galactic nucleus at these far-IR wavelengths comes from relatively cool dust a few parsecs from the centre and created by stars in the nuclear region. Two peaks are seen in this radiation, separated by about 1 arc min and on opposite sides of the centre along the galactic plane¹¹. There is a minimum in the far-IR radiation directly towards the centre which indicates a relative lack of dust within a radius of $\sim 1\ \text{pc}$ about the centre; radiation pressure from the bright nuclear radiation sources may have swept the gas and dust from their immediate vicinity. IR radiation from the more extended dust allows a rather good determination of the total luminosity of the galactic centre, as most of the luminosity is absorbed by dust and reradiated in the IR. It gives a luminosity of the central parsec between 10^7 and 3×10^7 solar luminosities ($L_\odot$).

Gas

Radio emission near the galactic nucleus is produced in part by relativistic electrons interacting with magnetic fields (synchrotron radiation) in a nearby supernova remnant, Sgr A East, and in part by bremsstrahlung of electrons and protons in ionized gas, of temperature about $10^4\ \text{K}$, in Sgr A West. In addition, an intense and puzzling 'point' source has been detected near the very centre. Radio observations of the Doppler widths of hydrogen recombination lines show that the ionized gas has rotational or turbulent velocities as high as several hundred km s^{-1} (ref. 12).

Recently radio interferometers have become available which can spatially resolve the structure of radio radiation from Sgr A West. However, the first observations of both the motion and detailed distribution of ionized gas were not made at radio wavelengths but in the IR, using emission lines of Ne^+ , or $[\text{Ne II}]$, at a wavelength of $12.8\ \mu\text{m}$ (refs 13–15). The ionized gas was found to be concentrated in small clouds which are

coincident with the concentrations of warm dust seen in Fig. 3. The $[\text{Ne II}]$ spectra of several concentrations are also shown. Emission to the right of the vertical axis in these spectra is from gas moving at positive (receding) velocities, that to the left at negative (approaching) velocities. There is a tendency for positive velocities to be found towards the north-east (upper left) and negative velocities towards the south-west, consistent with the galactic rotation, although chaotic motions are also seen. In the central $\sim 10\ \text{arc s}$, the axis of rotation appears to change, and is nearly perpendicular to that of the Galaxy. Recently the distribution of radio emission (Fig. 4) has been observed with the Very Large Array (VLA) of radio telescopes¹⁶. This determination of the distribution of ionized gas agrees quite well with that from the $[\text{Ne II}]$, as well as with the dust distribution indicated by $10\text{-}\mu\text{m}$ continuum observations.

The $[\text{Ne II}]$ observations show 14 clouds within $1.6\ \text{pc}$ of the centre. Their internal velocity dispersions are $\sim 100\ \text{km s}^{-1}$ whereas the average cloud velocities range over $\pm 260\ \text{km s}^{-1}$. Each contains a mass of about $1M_\odot$ within a diameter of $\sim 0.3\ \text{pc}$. The internal velocities, which are large enough to overcome any known force holding the cloud together, imply rapid expansion, and this in addition to their rate of collision with each other allows each cloud to exist for only $\sim 10^4\ \text{yr}$. If what is presently seen in the galactic nucleus represents a steady state situation, then a new cloud must be generated about every $10^3\ \text{yr}$. Both the cloud generation and the fate of gas left by the dispersed clouds, in amount $\sim 10^{-3}M_\odot\ \text{yr}^{-1}$, open interesting questions. Further information on this region has been provided by observation of IR fine structure lines of $[\text{Ar II}]$ (refs 17, 18), $[\text{Ar III}]$ (ref. 15), and $[\text{O III}]$ (refs 19, 20). Observations of $[\text{O III}]$ show that regions between the gas clouds mapped in $[\text{Ne II}]$ are largely empty, with densities no greater than $40\ \text{ions cm}^{-3}$.

To ionize the clouds observed radiatively, a source of 10^{51} ionizing photons s^{-1} is required. However, the observations of $[\text{Ar II}]$ and $[\text{Ar III}]$ indicate a low abundance of Ar^{2+} relative to Ar^+ , and hence a very limited number of photons which can

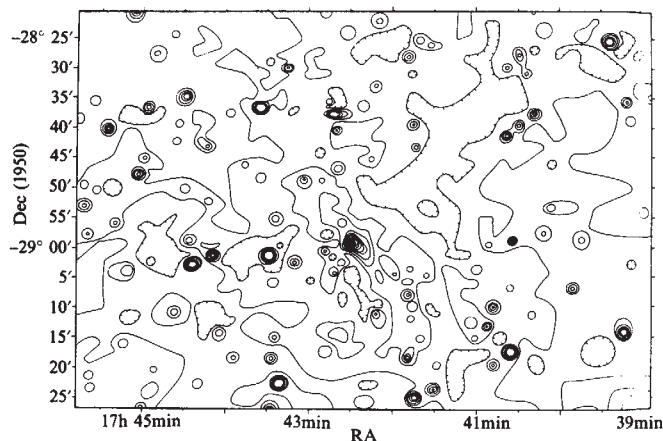


Fig. 1 Map showing intensity contours of $2.2\ \mu\text{m}$ radiation from the central 1° of the Galaxy⁴.

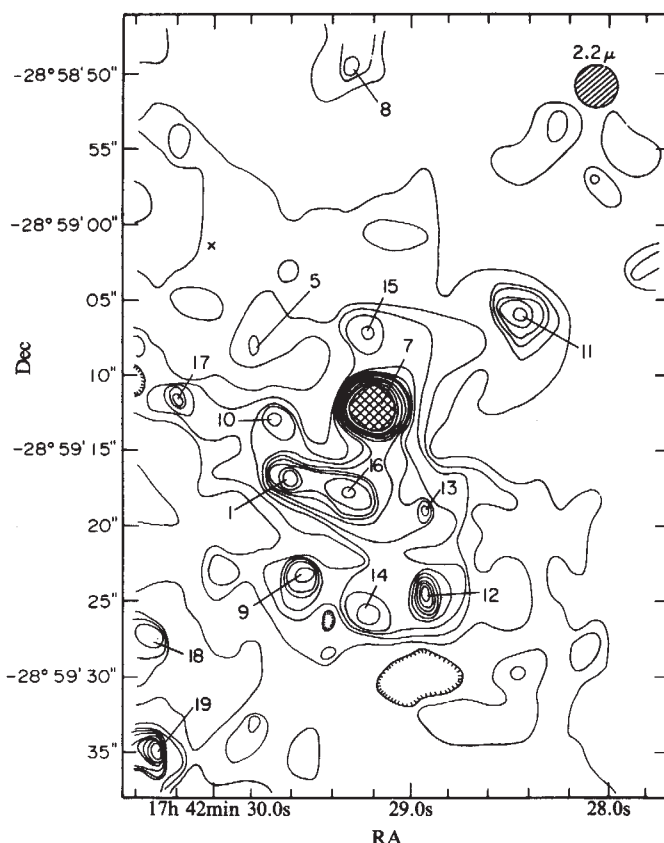


Fig. 2 Map of $2.2\text{-}\mu\text{m}$ intensity contours from the central 1 arc min of the Galaxy⁵.

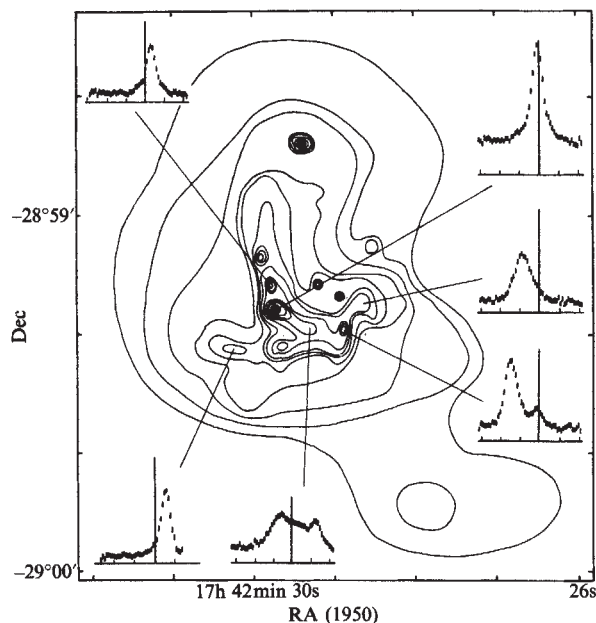


Fig. 3 Map showing intensity contours of 10- μ m continuum radiation from the galactic centre¹², with representative [Ne II] spectra¹⁵ from various 10- μ m peaks. The central vertical axis of each spectrum is at $V_{\text{LSR}} = 0$, and the horizontal axis is marked in 200 km s⁻¹ intervals.

ionize Ar⁺. This implies a rather cool ionizing source; no stars with surface temperature $\geq 35,000$ K can be present. Approximately 100 main sequence stars of effective temperature 35,000 K would be required to produce the total ionizing photons. Elsewhere in the Galaxy, clusters of ionizing stars contain a distribution of stellar temperatures, with most of the ionizing photons emitted by stars of $\sim 40,000$ K or hotter. Hence, an unusual object or an unusual collection of stars is required to explain the ionization of the galactic nucleus.

The distribution of cloud velocities may provide the most direct information on the mass distribution in the galactic centre. If, for example, they have circular orbits around the nucleus, the mass inside an orbit is easily calculated from the velocity and the gravitational constant. For more general orbits, the virial theorem may be used. It requires that the mean squared velocity of a test mass in the gravitational field of a mass distribution be $v^2(r) = GM(r)/r$, where $M(r)$ is the total mass inside a radius r and the average is either over an entire orbit or equivalently over a large number of test masses. The determination of $M(r)$ from the observed (instantaneous) cloud velocities has several problems. Only one component of their velocities and two components of their distances from the centre are measured so one does not obtain any complete picture of the orbits. If, for example, the clouds have just recently been ejected from a central object, the ensemble average of v^2 would not resemble the time average around an orbit. In addition, if the force of radiation pressure applied to the clouds is comparable with gravitational forces, their velocities would not reflect gravitation alone. In spite of these possible difficulties, it seems likely that the cloud velocities do provide a good estimate of $M(r)$. The most probable mass distribution derived from cloud velocities includes a distributed mass, that is, a stellar cluster, with $M(r) \approx 3 \times 10^6 M_{\odot}(\text{pc})$, in addition to a central concentrated mass of $\sim 3 \times 10^6 M_{\odot}$ (ref. 15). However, because of the small number of clouds observed, the statistical significance of the separation into a concentrated and a distributed mass is not high. For example, a mass distribution of $M(r) \approx 10^7 M_{\odot}(\text{pc})$, characteristic of a stellar cluster with no central massive object, is also allowed with a relative likelihood of $\sim 15\%$.

Another, completely different interpretation of the ionized gas clouds has recently been proposed by Brown²¹. He notes that the distribution of radio emission, shown in Fig. 4, is rather

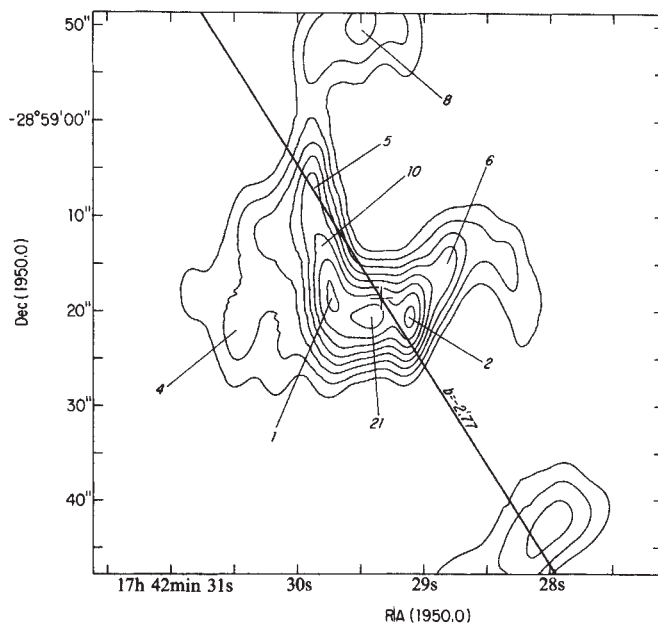


Fig. 4 Intensity contours of microwave radiation ($\lambda = 6$ cm) from the central 1 arc min of the Galaxy¹⁶. The diagonal line represents the plane of the Galaxy's disk.

similar to that obtained from [Ne II] observations but more S-shaped, with the centre of the S approximately at IRS 16, or the compact radio source. Brown interprets this distribution, and the velocities found from the [Ne II] line, as due to two oppositely oriented precessing jets of material emanating from the compact radio source with a velocity of 300 km s⁻¹ and a precession period of 2,300 yr. This behaviour is reminiscent of jets from the centres of more distant galaxies, or the rotating jets of gas associated with the peculiar object SS433 (ref. 22). However, the jet velocity in this case is very much slower than in other known cases, and the jet emission would need to be sporadic to generate discrete clouds. The proposed fit to the distribution of velocities does not seem especially close and formation of the typically rather circular clouds by jets is improbable. However, if true, interpretation of velocities in terms of the virial theorem and a mass distribution would, of course, be very misleading. The virial theorem interpretation does give masses which are quite consistent with those estimated from luminosity observations, and hence the total mass near the galactic centre is unlikely to be very different from that indicated.

The galactic nucleus is surrounded at some distance by clouds of neutral gas mixed with dust. Early continuum IR and microwave spectral line observations revealed dense clouds at distances of ~ 100 pc which are rich in warm dust and molecules. At these distances the clouds do not interact with the galactic nucleus strongly enough to tell us much about processes at the galactic centre. Recently, Lester *et al.*^{20,23} have detected oxygen atoms in neutral gas within about 6 ly (light year) of the nucleus, immediately outside the ionized region. This is the region where the two peaks of far-IR radiation from dust had been found. Molecules in the gas associated with this dust are at least partially dissociated by UV radiation from the central region, but the UV radiation is not intense enough to produce a large amount of ionization. The [O I] distribution, and velocities determined by Doppler effects, indicate a rotating disk of gas more or less in the plane of the Galaxy, but perturbed from a simple rotational pattern. Any deviation from simple rotational motion should be damped out after a few times the rotational period, about 5×10^4 yr. Hence, this appearance of noncircular motion indicates either some rather recent infall or outflow of material, or perhaps a nonspherical mass distribution in the galactic centre. I. Gatley and H. Hyland (personal communication) have recently detected rovibrational lines of H₂ in these same regions, near the peaks of far-IR dust radiation, indicating

the possible presence of shock waves from irregular motions of high velocity gas.

Peculiar objects

Most of the radiation from the galactic nucleus can be identified directly with stars, dust or gas. There are, however, a few sources whose nature is not yet clear. Radiation at X-ray and γ -ray wavelengths has been detected from regions near the galactic centre, as has an intense and varying source of 511 keV γ rays due to electron-positron recombination and annihilation. These suggest some peculiar object in or near the galactic centre. So do two other observations already mentioned briefly, a radio 'point' source and the peculiar, possibly related, small IR source IRS16.

It has been known since 1974 that within Sgr A lies a compact radio-emitting object²⁴⁻²⁶. This object is responsible for a few per cent of the radio emission from Sgr A at centimetre wavelengths. Its luminosity and behaviour make it difficult to classify; it is one or two orders of magnitude brighter than most other galactic compact radio sources such as pulsars or close binary stars, but many orders of magnitude weaker than the intense compact radio sources observed in a small number of extragalactic nuclei. The latter allows us no assurance of similarity with other galactic nuclei but is not necessarily surprising, because a similar source in other, more distant, galaxies would be too weak for detection. In its flat spectrum and slow variability, it does resemble some extragalactic radio sources more than other compact sources in our own Galaxy. The detailed structure of the source is not well known, although there is a likely upper limit to its size of 0.001 arc s (corresponding to 10 AU), which makes it the smallest known radio source in a galactic nucleus (again not surprising because this size would be unmeasurable in more distant galaxies).

The compact radio source is, within the uncertainty of 1 arc s, coincident with a near IR continuum source, IRS 16. Becklin and Neugebauer⁵ suggest that this source, which appeared slightly extended with an aperture of 2.5 arc s and is located near the centre of the more broadly distributed near IR emission, is the position of the highest stellar density in the Galaxy. IRS 16 has a colour like that of the nearby late-type giants, but shows much weaker 2.3- μ m carbon monoxide absorption, indicating that if it is a stellar cluster, it consists of rather early-type stars.

Two recent observations provide further information about the usual nature of IRS 16. First, Hall *et al.*²⁷ found very broad (FWHM = 1,500 km s⁻¹) He I (2.06 μ m) emission from the direction of IRS 16. However, it is remarkable that they and others have not found hydrogen emission lines to be broader than expected ($\sim$ 200 km s⁻¹) from the [Ne II] observations. They suggest that there is some central high velocity region, perhaps surrounding a massive object, in which hydrogen is severely depleted. Storey and Allen²⁸ have resolved IRS 16 into three components along the galactic plane, with $\sim$ 1.5 arc s between each; they found that the north-east and south-west components are associated with ionized gas, but the central one is not. They also determined that the 1- μ m sources detected earlier⁶⁻⁸ are not coincident with any of the 2- μ m sources. The compact radio source appears to be closest to IRS 16 SW, but could lie on one of the other nearby sources.

The radio and IR maps can be used to determine the centre of luminous material (excluding individual bright stars) in the nucleus. In addition, motions of the ionized gas as determined from the [Ne II] line measurements can be analysed to determine the dynamical centre of the Galaxy. The estimates agree that, to within a few arc seconds and within the uncertainties, IRS 16 and the compact radio source lie at the centre.

A broad spectrum of photons from a few keV energy to as high as 100 MeV has been found to come from the general region of the galactic centre²⁹. X rays of energy between 1 and 4 keV arise from a source 1 arc min or less in size and within 1 arc min of the galactic centre³⁰. However, they are relatively weak and not remarkable by comparison with X rays from

other sources in the Galaxy. Between 80 and 180 keV, the galactic centre source (or one within 0.5° of the centre) is relatively brighter, though not brighter than some other nearby sources²⁹. X-ray intensity from the direction of the galactic centre varies with time on a scale of about 3 yr, indicating that, regardless of whether the source is exactly at the galactic centre, it is relatively small—not more than 3 ly in diameter. At 10–100 MeV, γ radiation from the direction of the galactic centre is still more intense relative to other sources²⁹, although this energetic radiation has not yet been shown to originate closer than 1° from the centre.

Perhaps the most unusual radiation of all detected from the direction of the galactic centre is 0.511 MeV photons due to electron-positron annihilation. This was first detected, with poor spatial and spectral resolution, by Johnson and Haymes³¹ in 1973. Since then results have been refined by successive balloon or satellite experiments to show that the source is centred within a few degrees of the galactic centre, that the photon spectrum is not only clearly that of annihilation radiation, but most likely from positronium, and that the source is varying on a time scale of months indicating a size $\leq$ 0.1 ly or 10¹⁷ cm (refs 32–34). The intensity of the source has corresponded to annihilation of as much as 10¹⁶ g s⁻¹ of electrons and positrons. After 8 yr of such intensity, the source has recently become at least 10 times fainter, too weak to observe with present equipment³⁴.

A model of the galactic centre

Relatively direct inferences from the observational data present a context from which to model the extraordinary phenomena associated with the galactic centre. Apparently, the central 10 ly contains $\sim$ 10⁷ stars, a star density some 10⁷ times that of the solar vicinity. Interspersed in this dense stellar distribution we presently identify 14 ionized clouds of gas, each moving at velocities of $\sim$ 200 km s⁻¹, expanding at $\sim$ 100 km s⁻¹, extending $\sim$ 0.5 ly in radius, and containing about 1 M_⊙ of gas and a small percentage of dust. No gas or dust has yet been unambiguously observed in regions between these clouds, and clearly the density between them is very low. The expansion of the clouds would make them too diffuse to observe in 10⁴ yr, leading to the conclusion that a new cloud must be created about every 10³ yr. The gas is photoionized by an astronomically unusual radiation field with a high ratio of ionizing luminosity to effective temperature.

The unusual phenomena inferred from these results include the processes of creation, ionization, and disposal of the expanding gas clouds. One global model of the galactic centre which provides mechanisms for these three processes involves the creation of most of the clouds by the disruption of red giant stars, the ionization of the clouds by an unusual distribution of young, massive, hot stars, and the disposal of the expanded gas by intermittent radiation pressure and the gravitational collapse of the gas to form new stars³⁵.

Several processes might produce clouds of gas with characteristics like those observed. Their characteristics are quite different from those of normal H II regions, and it has been shown that they cannot be explained as modified planetary nebulae. The most plausible process suggested so far is collision of a red giant with a main sequence star. These collisions strip the loosely bound outer envelopes from the red giants and are likely to produce gas clouds of the required masses. After such a collision, the freed gas will have an expansion velocity comparable with that required for escape from the surface of a red giant, or $\sim$ 100 km s⁻¹—just that observed in the clouds. Collisions between more normal, main sequence stars, on the other hand, would free an insufficient cloud mass from the stars and also produce excessive cloud expansion velocities—near 1,000 km s⁻¹.

Once created from the disruption of red giants, the gas clouds expand and are eventually ionized throughout by the intense UV radiation field which is present in the galactic centre. In the present model, this radiation is assumed to be produced by

a distribution of young massive stars (OB stars) whose surface temperatures are all between about 30,000 and 35,000 K. In the solar vicinity, such a collection of OB stars would normally include stars hotter than 35,000 K. Stars of these high temperatures are evidently not present in this case, but several mechanisms possibly operant in the galactic centre could remove this normal hot component. Main sequence stars evolve by expansion and a concurrent drop in effective surface temperature. The hottest, most massive stars evolve most rapidly. A burst of 'normal' OB star formation 3×10^6 – 10^7 yr ago in the galactic centre would have evolved in time, allowing the hottest stars to cool until at present, no star would remain with a surface temperature exceeding 35,000 K. We shall discuss below some indirect evidence for sporadic activity in the galactic centre on this time scale. If, on the other hand, star formation in the galactic centre occurs more or less continuously, the deficiency of very hot stars must have another cause. There is evidence^{18,35–37} that the abundances of elements heavier than H and He increases towards the galactic centre, due to the rapid rate of star formation and therefore nucleosynthesis in this region. Stars which form from a medium richer in heavier elements than that in the vicinity of the Sun may have a different mass and temperature distribution, for example with very hot stars much rarer than normal^{38–40}. This possibility is supported by observations of ionized regions in the galaxy M101, which indicate that the effective temperatures of the ionizing OB stars decrease towards the centre of M101 as the heavy element abundance increases⁴¹. Similar measurements of the Galaxy have been hampered by the inability to observe at visible wavelengths into its inner regions.

It has been suggested by Lake and Norman⁴² that the central stellar cluster may have a triaxial rather than a spherically symmetric distribution, giving the galactic centre a structure like that seen on a larger scale in the barred spiral galaxies. Such deviation from a spherical distribution may be expected in view of the systematic velocity associated with the ionized clouds, since a triaxial distribution should occur if the kinetic energy associated with an average rotation is 0.14 or more of the total kinetic energy⁴³. Rotation of a triaxial distribution, or possibly oscillation of the central part of the stellar cluster in the gravitational minimum, may be coupled strongly enough to the immediately surrounding clouds to produce the apparent non-spherical motion of neutral gas at a distance of 10–30 lyr. It may also produce the variation of velocity with distance from the centre which is apparent in the ionized clouds.

The above model explains the distribution and motions of ionized and neutral gas in the galactic centre region totally within a framework of stellar interactions and stellar radiation fields of the inferred dense stellar distribution. However, it does not easily explain the large amount of material which may have been ejected from the galactic centre¹. Furthermore, the peculiar circumstances which produce the 'point' radio source and the electron-positron annihilation radiation would also have no explanation from such a model. Finally, the distribution of cloud velocities is more indicative of a massive ($3 \times 10^6 M_\odot$) point mass at the galactic centre than of a stellar cluster alone. These strongly suggest careful consideration of the possible presence of a massive black hole.

Evidence for a massive black hole

Lynden-Bell⁴⁴ first hypothesized that our galactic centre may contain a massive black hole by arguing that many galaxies go through a brief quasar phase in their youth, which dies out as the gas accretion rate onto the black hole dwindles. Therefore, the galactic centre may contain a 'dead quasar', a massive black hole into which some residual gas still accretes. The far-IR continuum observations of the galactic centre limit the current luminosity of a central source to less than $3 \times 10^7 L_\odot$ (ref. 11), while the [Ne II] (12.8 μm) spectroscopic observations reveal gas motions which apparently imply a central mass of $3 \times 10^6 M_\odot$, if one exists there¹⁵. Even a massive black hole as 'small' as $3 \times 10^6 M_\odot$ is, in principle, capable of producing

luminosities of $10^{11} L_\odot$, which would blow away the surrounding dusty gas by radiation pressure. Therefore, one might look for evidence for sporadic luminous bursts in the galactic centre. Oort¹ has reviewed the possibility of such events in the past and points especially at the observed clouds of molecular and atomic gas which appear to be radially expanding from the galactic centre. Typically, they are between 300 and 3,000 pc from the centre and have speeds of the order of 100 km s^{-1} , indicating they may have left the centre some 10^7 yr ago. Although other explanations may suffice (such as the burst of star formation postulated earlier for the source of ionizing radiation), the radiation pressure due to a luminous burst from a massive black hole might have propelled these clouds.

Other indirect, but still inconclusive, evidence for a massive black hole at the galactic centre include: the mysterious radio source, IRS 16, the X-ray source, and the electron-positron annihilation radiation, all of which appear to originate close to the centre of the Galaxy and may be associated with a black hole³⁰. However, the radio source, IRS 16, and the X-ray source can also be explained within the context of more familiar stellar objects observed elsewhere in the Galaxy^{30,45}. The annihilation radiation is unique so far as present astronomical observations are concerned and possible mechanisms for its generation near a black hole have been discussed^{46,47}. While this radiation requires a very unusual situation, and considerable progress has been made in analysing characteristics where it must be formed⁴⁸, the source cannot be tied in any unambiguous way to the existence of a black hole. As noted above, motions of the observed gas clouds point to the presence of a central object of mass $3 \times 10^6 M_\odot$, but the finite number of clouds available only give this a probability of 0.85.

One might adopt the view that, with so many partial indicators of a black hole, the case becomes convincing even though no one indication is conclusive. However there are also some negative indications. The case for a massive black hole would be greatly strengthened if its presence were shown to explain naturally the creation, ionization and/or disposal of the extraordinary expanding gas clouds. However, the black hole can account for none of these phenomena and, in fact, tends to eliminate the promising cloud creation mechanism of stellar collisions. Moreover, its presence could result in an excessive central luminosity produced by the rapid tidal disruption and inevitable accretion of the surrounding stellar population.

Stars orbiting about a black hole will occasionally approach closely enough to be disrupted by tidal forces. In that case, part of the stellar mass would orbit or fall into the black hole while the remainder would be accelerated away as an elongated gas cloud, with velocity dispersion of a few thousand km s^{-1} (ref. 35). Such a cloud is very different in shape and velocity dispersion from those observed. In the simplest model, stellar material captured by the black hole would form an accretion disk and provide a large luminosity as it gradually spirals inward.

The black hole cannot now be disposing of gas through accretion at the same rate as the gas is currently observed to be created. The resultant luminosity would be $> 10^8 L_\odot$. Nor is the observed luminosity presently sufficient to drive the gas and dust from the centre by radiation pressure. For the luminosity to be as low as is observed, we must be experiencing a lull in accretion activity. However, such a sharp lull is in doubt because at least the simpler models of accretion disks allow only a slow decay of luminosity after accretion. Hence, to maintain a black hole luminosity less than the current value of $3 \times 10^7 L_\odot$, no more than one tidal disruption every 10^4 yr can be allowed^{35,49,50}, which restricts the spatial density of the predominant main sequence stars. The red giant star density is fixed by the 2.2- μm continuum observations. From this, it can be shown that if a massive black hole is assumed the resultant overall stellar population is insufficient to provide the required rate of cloud creation through collisions of red giants with main sequence stars. Creation of clouds by the black hole tidal disruption of stars cannot replace the star-collision mechanism. According to the model described briefly here, it occurs too

infrequently and results in excessive cloud expansion velocities. One must note, however, the interesting spectroscopic evidence²⁷ for gas near IRS16 with velocity of $\sim 2,000 \text{ km s}^{-1}$, as would be expected from stellar disruption by a black hole. Additional clear-cut evidence of the existence of such gas would be important. Furthermore, if Brown's interpretation of the clouds as evidence for two jets emanating from the centre²¹ becomes convincing, most of the above arguments would no longer apply and a black hole could be favoured.

The assumption of a massive black hole also seems to fail, unfortunately, to provide the unusual ionizing radiation field. For a single source to ionize both the nearest and the most distant clouds to the observed degree, the effective temperature must be $\sim 31,000 \text{ K}$ and the total luminosity $\sim 8 \times 10^7 L_{\odot}$. Not only does this luminosity exceed observational bounds, but the $2.2 \mu\text{m}$ continuum from such a source would be at least an order of magnitude brighter than any individual source observed at the galactic centre³⁵. Hence, a black hole could be responsible for at most only a modest part of the ionizing radiation and provides no satisfying solution for the unusual radiation field.

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The question whether a black hole exists in the galactic centre must be left with an unsatisfactory answer. There are many phenomena which suggest a black hole but none which definitely establish its presence. Some observations are inconsistent with a black hole behaving in accordance with standard theories, and inconsistent with a black hole currently playing very much of a role in a broad range of phenomena we observe in some detail. However, the activity associated with black holes clearly can vary with time; it may have been dominant in this region in the past and is now quiescent. Also, the behaviour of a black hole may be much more complex than some of the standard theories presently allow⁴⁶, and in the face of these many complexities we have no real experience to help understand just what to expect. Hence, in spite of the remarkably detailed information about the galactic centre which has been acquired, intensive observations and theoretical analysis must continue before we know whether a black hole, or perhaps some other as yet unimagined object, is hiding in the unique spot which is the centre of our Sun's orbit in space and of our Galaxy.

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ARTICLES

Crater numbers and geological histories of Iapetus, Enceladus, Tethys and Hyperion

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Tethys, Iapetus and Enceladus, satellites of Saturn, display surfaces which indicate that geological processes have been active suggesting a certain degree of internal evolution. On Iapetus activity seems to have been confined to the dark terrain and its timing and extent remain unknown. The widest variation of terrains and crater numbers occurs on Enceladus and indicates the most prolonged geological activity of any satellite. Activity on Tethys seems confined to the first few hundred million years. The satellite Hyperion and the co-orbitals 1980S1 and 1980S3 show no geological activity and apparently are fragments of once larger bodies.

VOYAGER 2 provided the first high resolution observations of Iapetus, Enceladus, Hyperion and Tethys¹. As Saturn's satellites are intermediate in size and density between the galilean

satellites and asteroids, they represent a unique group of objects—small icy satellites. Understanding the geological history of these objects requires an estimation of the relative ages

Table 1 Crater number data (craters per $10^6 \text{ km}^2 \geq \text{diameter}$)

	Terrain	Crater diameters:						Counting area (km^2)
		5 km	10 km	20 km	30 km	40 km	50 km	
Iapetus	1		3,300 $\pm$ 150	500 $\pm$ 59	200 $\pm$ 37	95 $\pm$ 26	28 $\pm$ 14	146,090
	2		1,400 $\pm$ 116	330 $\pm$ 56	140 $\pm$ 37	99 $\pm$ 37	45 $\pm$ 21	104,370
	3		1,050 $\pm$ 124	380 $\pm$ 75	190 $\pm$ 53	130 $\pm$ 44	85 $\pm$ 35	68,355
	4		2,900 $\pm$ 211	680 $\pm$ 102	260 $\pm$ 63	170 $\pm$ 51	110 $\pm$ 41	65,240
	5		4,000 $\pm$ 210	620 $\pm$ 82	200 $\pm$ 47	130 $\pm$ 38	60 $\pm$ 26	91,140
	6		3,200 $\pm$ 168	740 $\pm$ 81	320 $\pm$ 85	150 $\pm$ 36	110 $\pm$ 31	113,930
	7		2,100 $\pm$ 157	560 $\pm$ 81	190 $\pm$ 47	150 $\pm$ 42	120 $\pm$ 38	85,416
	8		1,800 $\pm$ 115	410 $\pm$ 55	180 $\pm$ 36	100 $\pm$ 27	58 $\pm$ 31	136,680
Enceladus	Average		4,500 $\pm$ 75	740 $\pm$ 30	205 $\pm$ 16	110 $\pm$ 12	68 $\pm$ 9	811,210
	Cratered terrain (ct_1, ct_2)	4,200 $\pm$ 1,476	720 $\pm$ 349	330 $\pm$ 226	150 $\pm$ 159	85 $\pm$ 120	55 $\pm$ 96	12,895
	Cratered plains (cp)	4,700 $\pm$ 800	820 $\pm$ 334	170 $\pm$ 152	64 $\pm$ 93	35 $\pm$ 69	21 $\pm$ 53	7,343
	Smooth plains w/fractures (sp_1)	2,000 $\pm$ 628	350 $\pm$ 263	80 $\pm$ 125	31 $\pm$ 78	18 $\pm$ 60	10 $\pm$ 44	5,073
	Smooth plains (sp_2)	1,300 $\pm$ 355	175 $\pm$ 130	32 $\pm$ 56	12 $\pm$ 34	6 $\pm$ 24	3 $\pm$ 17	10,280
Tethys	Heavily cratered		2,000 $\pm$ 185	500 $\pm$ 92	225 $\pm$ 62	120 $\pm$ 45	80 $\pm$ 37	59,389
	Intermediately cratered plains		690 $\pm$ 72	170 $\pm$ 26	85 $\pm$ 25	45 $\pm$ 25	28 $\pm$ 14	133,960
	Plains		800 $\pm$ 66	140 $\pm$ 28	54 $\pm$ 17	37 $\pm$ 14	27 $\pm$ 12	185,520
	Ithaca Chasma		670 $\pm$ 117	120 $\pm$ 50	45 $\pm$ 30	28 $\pm$ 24	19 $\pm$ 20	49,221
Hyperion		5,000 $\pm$ 334	940 $\pm$ 135	150 $\pm$ 58	60 $\pm$ 37	30 $\pm$ 26	18 $\pm$ 20	44,731
Co-orbitals	1980S1		2,900 $\pm$ 485	650 $\pm$ 229	270 $\pm$ 148	130 $\pm$ 103	75 $\pm$ 78	12,361
	1980S3		3,000 $\pm$ 717	640 $\pm$ 331	260 $\pm$ 211	135 $\pm$ 152	80 $\pm$ 117	5,830
	1980S3		1,450 $\pm$ 608	210 $\pm$ 231	70 $\pm$ 134	34 $\pm$ 94	20 $\pm$ 72	3,925

of the various geological units observed on their surfaces. We present here the crater numbers—the number of craters of a given diameter or greater per 10^6 km^2 —for Enceladus, Iapetus, Tethys and Hyperion. Estimates of the absolute ages of those surfaces are based on a flux history model to be presented elsewhere. Crater numbers and preliminary geological histories for those satellites observed by Voyager 1 have already been reported².

Iapetus

Voyager 1 images of Iapetus³ revealed several large circular features which were highly suggestive of large impact craters. Higher resolution Voyager 2 images (Fig. 28 in ref. 1) indicated that the bright terrain is pock-marked with a myriad of large craters suggesting an ancient surface. Crater-like features were noted within the dark terrain near the albedo boundary, but most of the dark terrain appears featureless. It is unclear whether the dark terrain is actually featureless, or whether the contrast is so low that features are unobservable.

Crater numbers were determined only at large diameters because of low image resolution, 17 km per line pair (lp). Thus, it was impossible to determine the cratering record at small diameters or for small scale surface features. It was not possible to determine the dark terrain cratering record as no surface features were observable. A cumulative size-frequency distribution of bright terrain impact craters (shown in Fig. 1) represents an average of several smaller counting areas (Table 1). The bright terrain has 20- and 30-km crater numbers of 740 ± 30 and 205 ± 16 respectively. Linearly extrapolating to smaller diameters yields a 10-km crater number of $4,500 \pm 75$. The crater numbers for individual counting areas indicate that the bright terrain is uniformly cratered and that no major resurfacing has affected the terrain. Any resurfacing which has occurred did not affect craters ≥ 20 -30 km.

The pristine morphology of large (>100 km) craters suggests that significant crustal heating has not occurred. Any crustal heating would hasten the viscous relaxation of long wavelength crater topography⁴ as apparently happened to the large 400 km crater on Tethys¹. Presumably, the bright terrain crust has remained cold and rigid since formation $>3,800$ Myr ago. Dark material filling several craters within the bright terrain is the only visible modification. This may be an endogenic process

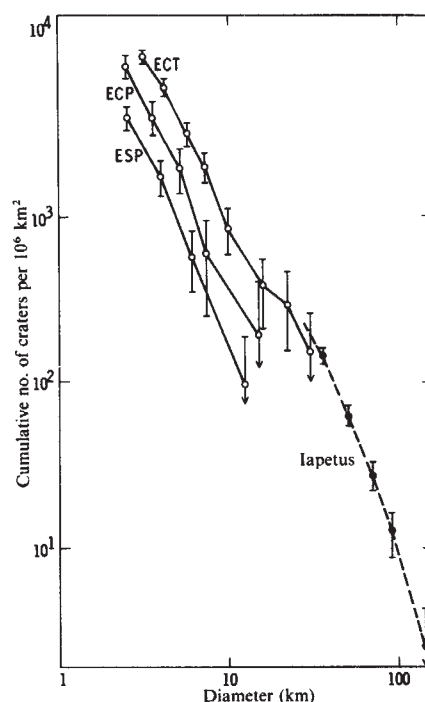


Fig. 1 Cumulative size frequency distributions for the bright terrain on Iapetus (dashed line) and several geological units of Enceladus (solid lines). For Enceladus; ECT, cratered terrain; ECP, cratered plains; ESP, smooth plains.

whereby dark material was erupted into the crater or an exogenous process whereby the material was 'blown in' from elsewhere.

Several theories have been advanced to explain the asymmetric albedo pattern. These fall into two broad categories: first, dark material was deposited on an originally bright surface from either an endogenous or exogenous source^{1,5}, and second, the darkening results from an erosion of a bright surface layer exposing a lower albedo substrate⁶. Smith *et al.*¹ feel the

evidence strongly favours the deposition theory but is insufficient to distinguish between an exogenous versus endogenous source for the material.

Soter⁵ suggested Phoebe as an exogenous source for the dark material. He speculated that material is eroded from Phoebe's surface, falls inward due to Poynting–Robertson effects and is swept up on the leading hemisphere of Iapetus. Such a scenario is troublesome. Ejection of sufficient material to darken Iapetus implies a high cratering rate at Phoebe. This suggests an ancient age as only during the heavy bombardment would a sufficiently high cratering rate occur. As the heavy bombardment ended, 3,800 Myr ago, several craters large enough to penetrate the dark blanket and expose the bright substrate should have formed, but such craters are not observed. This implies that either the blanket is in excess of several kilometres thick effectively preventing excavation by even the largest impact, or the blanket is so young that large craters have not had time to form. A relatively young age, rather than a significant thickness, may be indicated by the asymmetric albedo pattern. While Iapetus is currently tidally locked, its large orbital radius and low mass would allow appropriately oriented large impacts to break the lock. During the heavy bombardment the satellite was probably repeatedly spun-up. Low albedo material from Phoebe falling onto the spinning body would have been evenly distributed over the surface rather than asymmetrically as observed. A young age therefore appears to require an endogenic source. The dark material could have been generated internally and erupted through vents concentrated on the current leading hemisphere and subsequently ballistically distributed into the observed pattern by the micro-meteors.

Enceladus

The surface of Enceladus displays the widest variation of crater numbers and surface morphology of any saturnian satellite. Despite its relatively small radius (250 km), crater numbers indicate that it has the most prolonged geological history. Six terrain types have been identified on the body¹. These include cratered terrain (ct_1 , ct_2), cratered plains (cp), two smooth plains units (sp_1 , and sp_2), one of which is broken by rectilinear fractures (sp_1), and a ridged plains unit (rp). Crater numbers and cumulative size-frequencies have been compiled for these units (Table 1 and Fig. 1).

The number of 10-km craters for the heavily cratered terrain (ct_1 , ct_2) are 800–1,000. The cratered plains (cp) have slightly lower numbers at 400–500 and the fractured plains (sp_1) have numbers of only 100–200. Smooth and ridged plains units are devoid of craters to the image resolution limit (2 km lp^{-1}).

Shoemaker and Wolfe⁷ have proposed that a gradient in cratering rate from the apex (equator, 90° long.) to the antapex (equator, 270° long.) of motion occurs on tidally locked satellites. The ratio of the cratering rate at the apex to the antapex increases with decreasing orbital radii and is predicted to be >35 (ref. 1). To assess whether the observed variations in crater number are a reflection of only the gradient or if they indicate age variations, the 5-km crater numbers were plotted against their angular distance from the apex of motion (Fig. 2). The scatter of the data is inconsistent with the distribution expected from the gradient for a uniform age surface, the highest numbers at the apex sinusoidally decreasing towards the antapex. This suggests that the observed crater numbers reflect real ages and are not solely a function of geographical position. The sharp and well defined boundaries between geological units are also indicative of different ages as subtle rather than sharp boundaries would be expected from the cratering gradient.

The most heavily cratered areas on Enceladus appear coeval with the least cratered regions of other satellites, being $\sim 3,800$ Myr. The smooth and ridged plains, on which no craters were observed, have upper age limits of only a few hundred million years. Units with intermediate crater numbers fall between these two extremes. The uniformly low crater numbers and the absence of any observed craters >30 km indicates that little or no accretionary crust remains and implies that the entire

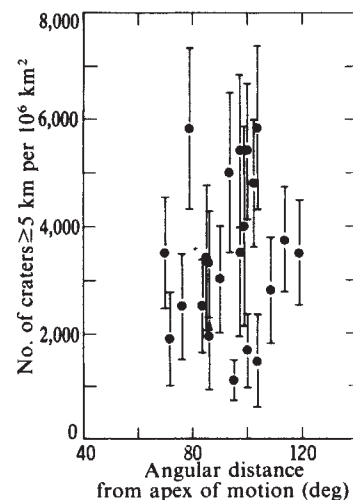


Fig. 2 Crater numbers, at diameters ≥ 5 km, for several counting areas on Enceladus plotted against their angular distance from the apex of motion (90° long., equator).

crust has been recycled. Enceladus has apparently been active over a much longer period of time and is presumably more evolved than the other satellites.

While statistically limited because of the small counting areas and low number of craters, the data seem to suggest that a continuum of ages is not represented by the various geological units, but rather each unit appears to represent a discrete period. This may be the result of intermittent rather than continuous resurfacing.

Tidal heating of Enceladus, induced by a 2:1 resonance with Dione, has been suggested as the source of energy driving the geological processes⁸. C. Yoder (personal communication) has proposed that tidal heating may be a periodic process. The eccentricity is first pumped up and the energy subsequently released through interior heating. Although not fully quantified, melting episodes and satellite reorientation may occur on time scales of 10^9 yr. During episodes of active heating, locally concentrated melting may occur at depth, migrate upwards and produce a young uncratered surface. Elsewhere, the increased heat flow would enhance the relaxation of crater topography. As the coefficient of thermal expansion of ice I is positive, internal heating of the satellite would cause a net increase in volume, either locally or globally, generating tensile stresses necessary to produce the rectilinear fracture patterns observed on some plains units. During periods of inactive tidal heating the surface modification processes would abate and cooling would occur. Such cooling should produce a contraction generating compressive stresses to form the ridges. Such a process would explain the apparent contradiction of young global scale features of both extensional and compressional nature. As craterless surfaces are involved this process must still be active, with the most recent resurfacing occurring perhaps within the past few hundred million years.

Tethys

Voyager 2 provided much higher resolution (5 km lp^{-1}) observations of Tethys (Fig. 25 in ref. 1) than did Voyager 1 (15 km lp^{-1}). The Voyager 1 images indicated that much of the satellite was heavily cratered^{2,3} and that a large fracture, Ithaca Chasma, was present. Voyager 2 data revealed a vast expanse of relatively young plains (the equator/300° long.), which together with Ithaca Chasma suggested some post-accretionary internal activity. Crater number data for several areas are displayed in Fig. 3 and Table 1.

Despite the age difference between the plains units and underlying cratered terrain, the crater numbers are very similar (Table 1). The number of 20 km crater on the plains is 170 ± 26 while that for the underlying intermediately cratered terrain is

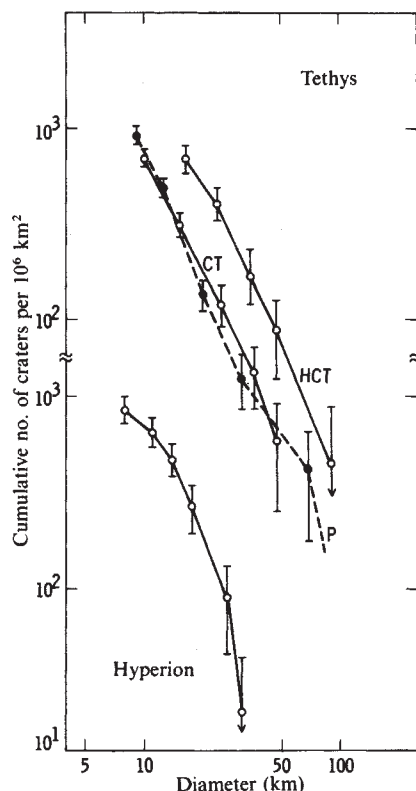


Fig. 3 Cumulative size-frequency distributions for various region of Tethys and for one part of Hyperion. For Tethys; HCT, heavily cratered terrain; CT, intermediately cratered terrain; P, plains units. Note the break in scale.

140 ± 28 . At larger diameters the two units have numbers which are not significantly different. As the large craters exposed on the plains actually protrude from the underlying more heavily cratered terrain. The small difference in 10-km crater numbers suggests a very short interval between the formation of the underlying cratered terrain and the plains. A much older expanse of heavily cratered terrain occurs near long. 60° with a 20-km crater number of 500 ± 92 . Crater numbers for the interior of Ithaca Chasma are similar to those for the adjacent areas with 20-km crater numbers of 120 ± 50 . The data show that the surface within the canyon is the least cratered and perhaps the youngest, which may indicate that Ithaca Chasma is the youngest tectonic feature on Tethys. However, as the statistical uncertainties are large this is only speculative.

Plains material apparently was deposited over a broad expanse of cratered terrain, burying the small and intermediate size craters but leaving the largest craters exposed. This incomplete burial has resulted in a cumulative size-frequency distribution (Fig. 4) with a bimodal slope. The large diameter (>25 km) segment ($m = -1.4$) represents the large unburied craters, while the small diameter segment ($m = -3.0$) represents those craters superimposed on the plains. The difference in size-frequency distributions suggests that each segment was produced by a different population of impacting debris.

Crater numbers (Table 1) suggest that Tethys has undergone some degree of internal activity producing surfaces of several ages. The most heavily cratered terrain, near long. 45° , is the oldest and dates back to the heavy bombardment $\sim 4,000$ Myr ago. Units with intermediate crater number, near 330° long., represent a slightly younger surface. The plains units are marginally younger than the intermediately cratered areas and presumably result from surface flooding by internally derived material. The crater number of the plains unit suggests that they formed $\sim 3,800$ – $4,000$ Myr ago. Thus, while Tethys experienced some activity after accretion it was probably short-lived, perhaps a few hundred million years.

Hyperion

Hyperion is relatively small and the only nonspherical of the principal saturnian satellites. Smith *et al.*¹ have suggested that the irregular shape has resulted from an impact-induced breakup of a once larger body.

Image resolution of Hyperion (9 km lp^{-1}) was sufficient only to allow the determination of the cumulative size-frequency distribution for relatively large craters. Craters in the diameter range of 20–50 km are common with one >100 km. Crater counts (Table 1 and Fig. 3) were determined from the highest resolution image (Fig. 27 in ref. 1) and the high crater number (20-km number = 150 ± 58) suggests that Hyperion's surface is relatively old. There is no evidence of internally driven resurfacing to suggest any internal evolution. The numbers are too low to indicate a surface age $>3,800$ Myr and the age probably represents the timing of the break up of a larger parent body. Subsequently the only processes affecting Hyperion's surface were smaller impact events.

Co-orbitals 1980S1 and 1980S3

Data from both Voyager 1 and 2 images^{1,3} for the co-orbital satellites have shown that at least two surface ages are present on 1980S3. Crater numbers (Table 1) for the most heavily cratered part of 1980S3 and for 1980S1 are identical and suggest an ancient, heavy bombardment age. One area of 1980S3 has distinctly lower crater numbers which may represent the event during which the two bodies were split apart. There are no indications of any resurfacing on either body which might suggest that they evolved internally.

Discussion

Similar to the satellites observed by Voyager 1, Enceladus, Tethys and perhaps Iapetus have developed to some degree beyond their accretionary states. In view of the small size and low density of these objects it is surprising that any evidence of geological activity is observed. Only Hyperion and the co-orbitals apparently remain completely unmodified by endogenous processes.

Consolmagno and Lewis⁹ have calculated thermal evolutionary models for small icy satellites of various sizes using a 60% ice/40% rock mixture. Assuming chondritic elemental abundances, satellites <500 km radius (Mimas, Enceladus, Hyperion) would remain entirely unmelted throughout their history. For a 700-km radius body (Rhea, Dione, Iapetus, Tethys) limited melting of the interior would occur, however 90% of the volume would still remain unmelted. Larger radius satellites (Titan) would undergo considerably more melting but regardless of size near-surface melting would not occur. The addition of 10% ammonia hydrate significantly alters the results in some cases. While a 500-km radius body will still remain frozen throughout its history, larger objects would undergo significantly more melting than with water as the only ice. Internal temperatures rise over the first 2,000 Myr and subsequently decay. Stevenson¹⁰, using similar elemental abundances, added a thick surface regolith and calculated a series of thermal models. The degree of internal melting is significantly increased due to the insulating effects of the blanket. However, neither complete nor near-surface melting occurs at any satellite size.

Other possible energy sources to drive internal activity appear insufficient. Peale *et al.*¹¹ have argued that tidal processes were unimportant contributors to the total heat budget of any satellite, although Enceladus appears to have experienced significant tidal interaction. Gravitational energy produced by differentiation would be limited and induce no activity significantly above that already produced by radioactive decay. It is possible that impact heating during the heavy bombardment could have been important in driving the observed resurfacing.

The observed geology indicates that there was an energy source and that significant internal evolution probably occurred. Clathrates and other non-water volatiles which enhance melting by lowering the melting temperature are clearly indicated. A

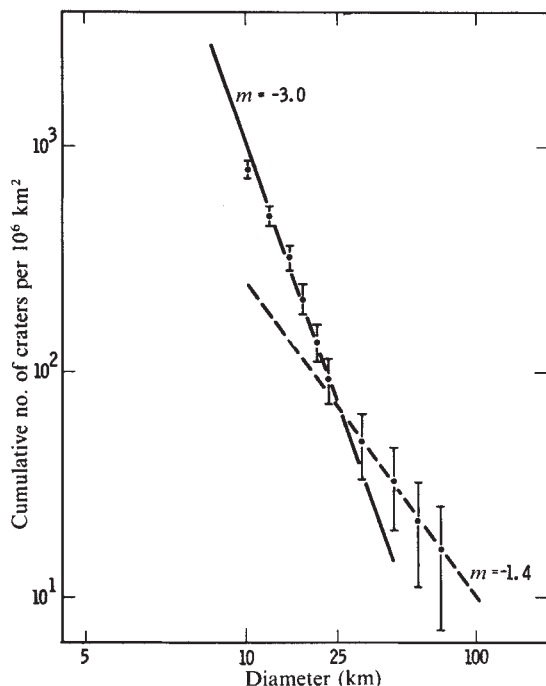


Fig. 4 The same size-frequency distribution for the smooth plains unit on Tethys used in Fig. 3 but illustrated alone for clarity shows the bimodal slope of the distribution and reflects two populations of impacting bodies. For $d > 25$ km, $m = -1.4$, for $d < 25$ km, $m = -3.0$.

problem remains in that no model predicts surface or near-surface melting. This implies either a mechanism whereby melted material at depths of tens to hundreds of kilometres is efficiently transported to the surface, or some undescribed process which can produce near-surface melting. Near-surface melting might be enhanced as the result of inhomogeneous accretion. Smith *et al.*³ have proposed that the lack of a density/orbital radii correlation may result from the satellites being composed of only a few protosatellite bodies. Those protosatellites with high silicate/ice ratios would have higher densities and vice versa. If such a silicate body occurred near the surface, its stored accretional and radioactive heat might enhance melting and thus drive local resurfacing. Conversely, deep-seated fractures from large impacts may provide conduits for deep-seated melts to erupt onto the surface.

The composition of the young plains units and its mode of emplacement are both uncertainties. Water ice, identified in the reflectance spectra¹²⁻¹⁴, is commonly assumed to be the dominate volatile and thus the material erupted onto the surface. However, the spectra are complicated and apparently indicate additional components. Clathrates of ammonia or other compounds have also been suggested to occur^{1,3,9,10}. Although such materials have lower melting temperatures and thus are more easily mobilized than water, they would probably be erupted in a gaseous rather than liquid phase. Such material

would condense and then fall back to the surface as a snow (ref. 1 and T. Owen, personal communication), producing a smooth young surface.

Enceladus, unlike the other satellites, appears to derive its energy through tidal interactions with Dione⁸. Although the effectiveness of the process has been questioned¹¹ it remains the only viable option. Tidal heating can provide energy episodically to the system indefinitely and on a local scale. Thus the surface of Enceladus can be continuously modified over geological time. The vast expanses of craterless terrain attest to the ongoing nature of the resurfacing.

All of the larger satellites, except Iapetus and Enceladus, exhibit surfaces bombarded by two groups of impacting objects producing Population I and II type craters. Population I is characterized by numerous large diameter craters relative to small craters, while Population II consists predominantly of smaller diameter craters, generally ≤ 30 km (refs 1, 3). These two populations are clearly discernible because of the significant differences in slopes, as shown in Fig. 4. Population I craters are represented by the large diameter craters on Hyperion, the bright terrain of Iapetus, and the heavily cratered parts of Tethys being formed during the late heavy bombardment. Population II craters are typically found on the plains of Tethys and all of Enceladus and postdate the heavy bombardment.

Tethys is generally an ancient body recording the late heavy bombardment. Resurfacing has affected a broad area of the trailing hemisphere at least twice. An initial resurfacing removed the most heavily cratered areas, typical of that near 45° long, forming a unit with intermediate crater number. Almost immediately thereafter a plains unit was emplaced, partially burying the older surfaces. The formation of Ithaca Chasma appears to be an ancient event.

The dark terrain on Iapetus apparently indicates that some type of internally driven activity has occurred. As no surface features were observed the nature of that process is unknown. The bright terrain has remained static since the formation of the body. The pristine large diameter craters and high crater numbers attest to the lack of resurfacing or significant crustal heating in those areas. If internal processes were active, their effects were limited to the current leading hemisphere.

Enceladus is the most evolved satellite comparable perhaps only to Io in terms of the level of dynamic activity. The oldest surfaces on Enceladus have only Population II craters suggesting that the entire body has probably been resurfaced at least once since the heavy bombardment. The youngest areas are craterless and probably no more than a few hundred million years old. Rectilinear fractures and ridges attest to the alternating episodes of global compression and tension.

Hyperion and the co-orbitals apparently are fragments of a once larger body. The fracturing of the parent body, in both cases, appears to have occurred near or just after the end of the heavy bombardment. Other than impact modification no resurfacing seems to have occurred.

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The structure of a mutant *H-2* gene suggests that the generation of polymorphism in *H-2* genes may occur by gene conversion-like events

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We have cloned a mutant allele of the *H-2K^b* gene known as *H-2K^{bml}*. DNA sequence analysis of this gene shows that it is the result of an intergenic exchange of DNA (gene conversion) which results in the conversion of a short internal segment of the *H-2K^b* to the corresponding sequence of another *H-2* gene. We suggest that this type of gene conversion is widespread in *H-2* genes and that it is a major force in the generation of polymorphism in *H-2* genes.

THE major histocompatibility complex (MHC) has an important role in the regulation of the immune response in vertebrates. The so-called class I MHC proteins are the classical transplantation antigens: in mouse *H-2K*, *D* and *L*, and in man HLA-A, B and C. These proteins appear to function by presenting foreign antigens such as viral proteins to cytotoxic T cells of the immune system and are found on virtually all cell types. The genes for the murine class I antigens are localized on chromosome 17; Fig. 1 is a schematic genetic map of the locus; it shows the class II (*Ir*) genes sandwiched between *H-2K* and *H-2D*. The *Qa* and *Tla* genes are localized telomeric to the *H-2D* gene and encode class I-related antigens which are expressed on certain classes of lymphoid cell. Both the class I *H-2K*, *D*, *L* antigens and the *Qa* and *Tla* antigens are integral membrane proteins of 40–45,000 molecular weight associated with β_2 -microglobulin, a protein of 12,000 molecular weight, not encoded in the MHC.

Molecular cloning of the *H-2* genes has shown that there are a large number of *H-2* related DNA sequences in the mouse and that, surprisingly, the majority are located in the *Qa2.3* and *Tla* regions^{1–4}. These studies also establish the high degree of homology between the classical class I genes and genes in the *Qa* and *Tla* regions.

An intriguing property of the *H-2* and HLA antigens is their extreme polymorphism. Allelic gene products from different inbred mouse strains differ extensively in amino acid sequence and hence in serological properties. About 50 different alleles at each of the *H-2K* and *H-2D* loci have been observed (see ref. 5). Most significantly, a given allele of, say, the *H-2K* gene is commonly no more similar to another allele than it is to a given non-allelic *H-2D* gene. This unusual genetic phenomenon has a precedent. Thus, Slightom *et al.*⁶ showed that a human γ -globin gene was more similar in DNA sequence to its linked γ -globin gene than it was to the allelic γ -globin gene found on the other homologous chromosome. They suggested that this resulted from a intergenic exchange during recombination (called here a gene conversion event) between the two linked γ - and γ -globin genes which caused the γ -globin sequence to be corrected against (converted to) the linked γ -globin gene⁶. These and other observations led us to suggest that similar gene conversion events could be responsible for the introduction of new sequences in a given *H-2* gene and hence for the generation of a high number of polymorphic alleles at the *H-2* loci in the mouse population; donor non-allelic *H-2*, *Qa* or *Tla* genes or pseudogenes could be the source of new information for incorporation at the *H-2* loci⁷. Others have made similar suggestions^{8,9}.

Ideally, to establish the mechanism underlying such a genetic event, it is desirable to have the DNA sequence of donor and recipient genes before and after the event. This is made possible by the fact that spontaneous mutants from inbred mouse strains exist which have altered *H-2* molecules and hence carry new polymorphic alleles. Therefore, if a gene conversion event has occurred both donor and recipient genes are available in the non-mutant strain so three of the four pieces of the puzzle can be determined.

We have previously cloned and sequenced the *H-2K^b* gene of the B10 mouse¹⁰. We have therefore chosen to clone and sequence the gene encoding a mutant form of this gene, *H-2K^{bml}*. The comparison of these sequences shows that a total of seven nucleotide changes have occurred in the *H-2K^{bml}* gene which cause three amino acid substitutions. These nucleotide changes are clustered in a 13-nucleotide region of the *H-2K* gene. We suggest that these mutations have been introduced into the *H-2K^b* gene by a gene conversion-like event and that these events have a major role in the generation of polymorphism in *H-2* genes.

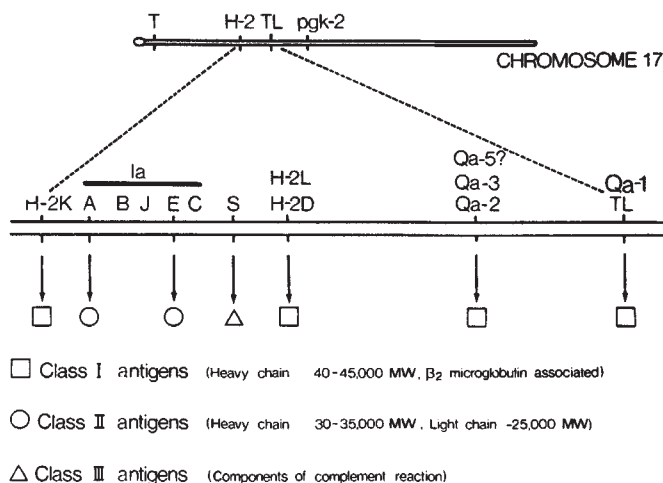
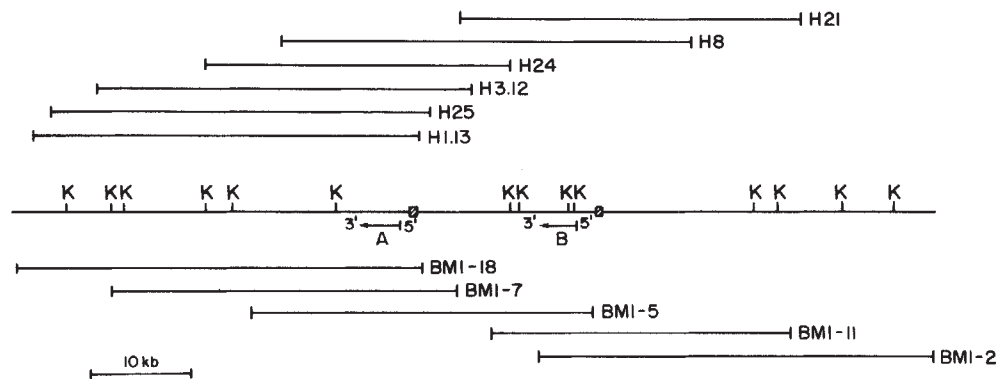


Fig. 1 Genetic map of the murine *H-2* and associated genetic loci. The top line shows a diagram of chromosome 17 with the centromere on the left. The bottom line shows an expanded diagram of the regions between the *H-2K* locus on the left and the *TL* locus on the right. *H-2* class I molecules (□) are expressed from at least four separate genetic loci as shown by the arrows below the line.

Fig. 2 A restriction enzyme map of the region containing the *H-2K^b* and *H-2K^{bml}* gene; with this enzyme the two regions are indistinguishable. The middle line shows the extent of the cloned region with the *KpnI* (K) restriction sites. Class 1 genes (A and B) are shown with arrows indicating the direction of transcription 5'→3'. The hatched boxes indicate the localization of the 5' flanking *Bam*HI fragment used for screening. Above the restriction map is shown the extent of the overlapping cosmids from the B10 library (H) and below are the overlapping cosmids from the B6 bml library (bml).



Isolation of the mutant *H-2K^{bml}* region

We have previously cloned the *H-2K^b* region from a cosmid library prepared from the B10 mouse⁴. We used the same general strategy to isolate this region from the B6^{bml} mutant. A cosmid library was prepared using procedures developed in this laboratory^{11,12} and screened with a probe from the 5' flanking sequences of the *H-2K^b* gene (see ref. 4). This probe detects about six DNA segments linked to *H-2* related genes from the *H-2K* and *Qa2,3* regions. The clones obtained were then examined by restriction enzyme digestion and Southern blot hybridization to detect the *H-2K^{bml}* locus. Five overlapping clones at the *H-2K* locus were obtained and restriction maps were determined. The general organization of this region is identical to that reported by us previously for the *H-2K^b* region⁴; two linked genes are seen as shown on the map of Fig. 2. That the bml mutation indeed resides within the *H-2K* gene previously identified⁴ was shown by subcloning this gene and transforming mouse L cells. The L-cell transformants were then used as target cells for allogeneic cytotoxic T cells derived from the *H-2^b* mouse (B10) and directed against spleen cells from the bml mouse (Fig. 3). These transformed cells are killed by cytotoxic T cells specific for bml but are not killed by cytotoxic T cells directed against the *H-2K^b* determinant. This result excludes the alternative explanation that the *H-2K^b* gene has become silent and that the *H-2^{bml}* antigen is expressed from another gene in the genome.

Comparative fine mapping studies were also performed on the subcloned *H-2K^b* and *H-2K^{bml}* genes (Fig. 4). Two differences were seen in exon 3 which encodes the second domain of the *H-2* molecule. First, a *HinfI* site is present in *H-2K^b* but absent in *H-2K^{bml}*; this site is present at the codons for amino acid residues 155 and 156 which are altered from Arg-Leu in *K^b* to Tyr-Tyr in *K^{bml}*, therefore this change is expected. In addition, a *PstI* site has been gained by the *H-2K^{bml}* gene just 5' of the *HinfI* site. These observations suggest that multiple nucleotide differences must occur between these two genes.

The DNA sequence of the *H-2K^{bml}* gene

We have therefore determined the DNA sequence of exons 1 to 6 of the *H-2K^{bml}* gene and segments of their flanking introns using the Maxam-Gilbert procedure and the strategy outlined in Fig. 4 legend. In total only eight nucleotide differences are evident. A single nucleotide difference exists in intron 1 where *K^b* has a T and *K^{bml}* has an A at the residue 116 nucleotides from the 5' end of the intron. This may be a polymorphic difference between the *H-2K^b* gene from these two closely related mouse strains (the *H-2K^b* gene is from B10 and the *K^{bml}* gene from B6). The remaining seven differences are clustered in a short region of 13 nucleotides of exon 3; two changes are present in the codon for amino acid 152 which cause an amino acid substitution from Glu (GAA) to Ala (GCT) and five changes at the codon for amino acids 155 and 156

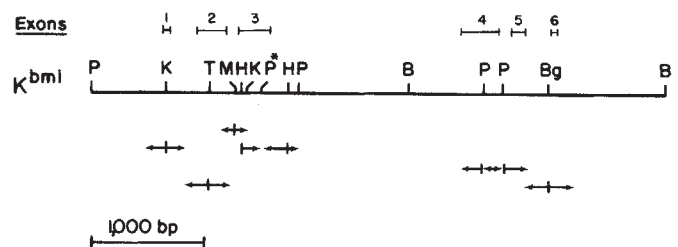


Fig. 3 Fine restriction map of the *H-2K^{bml}* gene and sequence strategy. The middle line shows a fine restriction map of the *H-2K^{bml}* gene. B, *Bam*HI; Bg, *Bgl*II; H, *Hinf*I; K, *Kpn*I; M, *Msp*I; P, *Pst*I; T, *Taq*I. Above the restriction map is shown the position of exon 1 (leader) and exons 2 (first domain), 3 (second domain), 4 (third domain), 5 (transmembrane part), 6 (first cytoplasmic part). The straight arrows below show the direction and length of the fragments sequenced with the Maxam and Gilbert procedure. *An additional *Pst*I site in the *H-2K^{bml}* gene, not found in the *H-2K^b* gene.

causing Arg 155(AGA)→Tyr 155(TAT) and Leu 156(CTC)→Tyr 156(TAC). The changes at residues 155 and 156 agree with the published data from the structural analysis of the protein but the change at residue 152 must have been missed in that study¹³. Our data show that these changes have occurred in the *H-2K^b* gene. We consider that a gene conversion event is the most likely cause for this phenomenon in which case there must be a donor gene which contains this novel nucleotide sequence. There are many candidates for such a donor gene in the mouse genome because estimates of the number of class I-related genes in the mouse genome range between 15 to 30 (refs 3, 14). Of the reported *H-2* class I sequences; *H-2K^d* (S. Kvist, personal communication), *H-2D^d* (ref. 15), *H-2L^d* (refs 9, 16), *H-2D^b* (ref. 17), clone 27.1 (ref. 1) and the *H-2*-like gene closely linked to *H-2K^b* (Fig. 5), *H-2L^d* is the only gene which contains the amino acids found in the bml sequence in this region. It is, in fact, possible that the *H-2L^d* gene was the donor gene as the bml mutation was first detected in a heterozygous (B6×BALB/c) F₁ mouse^{13,18}. In this case, the extent of the gene conversion event can be predicted (Fig. 5) and must be from 13–51 bases. If the *H-2L^d* gene was the donor gene, however, the gene conversion event must have taken place in the zygote. It is also possible that the donor gene was another class I gene or pseudogene in the B6 or BALB/c genome and which also contains the same nucleotide sequence in the region from residues 152–156. Further experiments will be required to exclude this. In addition, it is not formally excluded that the seven nucleotide changes seen here are independent point mutations, or a hot spot of nucleotide substitutions caused by a conventional base-substitution mechanism. We consider these unlikely; and the fact that the *H-2L^d* sequence in this area is identical supports this point of view.

Fig. 4 Comparison of the DNA and protein sequence of the *H-2K^b* gene, *H-2K^{bml}* gene, *H-2L^d* gene^{9,17} and the 'A' gene of gene region I (refs 4, 14) (see Fig. 2) starting from amino acid 145 to 168. The recognition sequences for the enzymes *Pst*I and *Hinf*I are underlined. Only the nucleotides and amino acid differences from *H-2K^b* are shown in the *H-2K^{bml}*, *H-2L^d* and 'A' gene.

	145	150	152	155 156	160	165	
<i>K^b</i>	HIS LYS TRP GLU GLN	ALA GLY GLU ALA GLU	ARG LEU ARG ALA TYR LEU GLU GLY THR				<i>K^b</i>
<i>K^b</i>	CAC AAG TGG GAG CAG	GCT GGT GAA GCA GAG	<u>AGA CTC</u> AGG GCC TAC CTG GAG GGC ACG				<i>K^b</i>
<i>K^{bm-1}</i>			CT	TAT TA			<i>K^{bm+1}</i>
<i>L^d</i>	G		CT	TAT TA		GA	<i>L^d</i>
<i>L^d</i>	ARG	ALA	TYR TYR		GLU		<i>L^d</i>
Gene A	A A CT	A T	TT	A GAG		G	gene A
Gene A	GLU TRP	VAL	VAL	GLU		ALA	gene A

Generation of polymorphism in the MHC

Non-allelic gene conversion is a likely explanation for the origin of the *bml* mutant. The limited amino acid sequence data available for additional *H-2* mutants suggest that this may turn out to be a general phenomenon. *H-2K^{bml}* and *H-2K^{bm9}* has two amino acid substitutions, namely Tyr 116→Phe and Cys 121→Arg. This requires a minimum of two nucleotide substitutions and interestingly Phe 116 and Arg 121 are already found in *H-2D^b*. Thus, a conversion using *H-2D^b* as the donor gene might be the source of the new information in *H-2K^{bml}*.

Likewise *bm5* and *bm6* have a single substitution at Tyr 116→Phe 116; again Phe 116 is present in *H-2D^b*. A second argument supporting gene conversion as the origin of many *H-2* mutants is the fact that the same mutation has been isolated in different experiments. This would be expected for a gene conversion event and would be unlikely for conventional base substitutions unless there would be a strong selection for these particular amino acid changes. It is striking that the seven base changes are clustered in a very short region of DNA in the *bml* mutant gene. Furthermore, the polypeptide sequences in the other mutants cited above suggest that short conversion events will be the rule rather than the exception.

We consider it likely that gene conversion may turn out to be the major force in the generation of new alleles of *H-2* genes. The mutants seen above are almost certainly part of the same genetic phenomenon that is responsible for the production of new *H-2* alleles in the wild mouse. The gene conversion discussed above must have occurred between non-allelic genes and conceivably between two different haplotypes and hence different chromosomes in a heterozygous mouse. Furthermore, we should expect that genetic exchanges between alleles will occur and these can potentiate the transmission of new information from a 'converted' allele to other alleles of the same gene within the mouse population. The fact that the *H-2K* and *H-2D* genes show little 'K-ness' and 'D-ness', at least in certain haplotypes, suggests that non-allelic genetic exchanges must have a profound role in the generation of polymorphism. It is interesting that while the classical *H-2K* and *D* genes show extensive polymorphism, there are only few alleles of expressed *Qa* and *TL* genes. If the *Qa* and *TL* genes participate in gene conversion events, then they do this either only as donors, or alternatively there is a strong selection eliminating the changes that have been introduced in the *Qa* and *TL* genes.

Is gene conversion in *H-2* genes more frequent than in other genes and, if so, is there anything unique about the structure of *H-2* genes to promote this? It is not possible to say whether the frequency in *H-2* genes is higher than, for example, in globin genes. Certainly polymorphism is rare in globin genes. We have sequenced four β -globin genes and found them to be identical in DNA sequence even in intron sequences which have little or no selection on their primary DNA sequence^{19,20}. Polymorphic forms have been detected but again the number of these is extremely limited. Gene conversion events have occurred in the globin (and immunoglobulin) gene families but these seem to be rare and should be considered over evolutionary time scales (see, for example, ref. 6). As far as unique structures in *H-2* genes are concerned, we are struck by the

high G+C content of *H-2* introns (greater than 90% near exons) compared with, say, globin genes where the introns are in fact A+T rich. Slightom *et al.*⁶ noted that the gene conversion event in the human γ -globin genes was associated with the presence of a short (about 40 nucleotides) CG: TG heteropolymer; the *H-2* intron sequences have some similarities with this

Table 1 Lysis of L cells transformed with cloned *b* and *bml* *H-2K* genes

Target cells	% Specific lysis Cytotoxic T cells	
	anti- <i>K^b</i> (<i>bml</i> anti-B10)	anti- <i>bml</i> (CBA×B10) anti- <i>bml</i>
B10.A(5R) (<i>K^b</i>)	28*	5
<i>bml</i> (<i>K^{bml}</i>)	-6	65*
LD-1	5	-6
Expt 1		
LD1/Bm5/G	6	20*
LD1/Bm5/H	-3	28*
LD1/Bm7/G		0
LD1/Bm11/G	TF	19*
LD1/Bm18/G		-3
LD1/Bm21/G		32*
LH8-2	21*	-4
Expt 2		
B10	56*	8
<i>bml</i>	6	77*
LD1/Bm7/G	4	1
LD1/Bm11/G	3	13*
LD1/Bm18/G	3	2
LD1/Bm21/G	3	24*
LH8-2	8*	1

$1-2 \times 10^6$ target cells were labelled with 100 μ Ci 51 Cr-labelled sodium chromate for 1 h then washed twice. 1×10^4 labelled target cells in 100- μ l volumes were placed in round-bottomed wells of microtitre plates. For cytotoxic T-cell lysis (CML) killer cells generated in 5-day mixed lymphocyte cultures of the strain combinations designated were added in 100 μ l to give attacker:target (A:T) ratios starting at 50:1 and thereafter at doubling dilutions of attackers giving four different A:T ratios in all. Three triplicate wells of each A:T ratio were set up. After 4 h incubation at 37 °C in a 5% CO₂ humidified atmosphere the plates were spun and 100 μ l of the supernatant from each well was collected and counted for released γ counts. The per cent specific lysis was calculated according to the formula

$$E - C/M - C \times 100\%$$

where *E* is c.p.m. for target cells incubated with killer cells, *C* is c.p.m. from target cells incubated with medium alone and *M* is c.p.m. from target cell lysis with Triton. The per cent specific lysis shown is for an A:T ratio of 20:1 and the value is taken from a 12-point regression analysis of the data obtained in the titration described above. LD1, control L cells. LD1 cells were transformed with cosmid DNA (BM1-5, BM1-7, BM1-11, BM1-18 and BM1-21) using the CaPO₄ mediated DNA transfer technique²¹. Population of transformed cells (Bm5, Bm7, Bm11, Bm18 and Bm21 respectively) were selected using G418 (G) or hypoxanthine-aminopterin-thymidine (H) selection and used as target cells in the CML tests described above. LH8-2, L-cells transformed with cosmid H8 containing the *K^b* gene (see ref. 4). TF, technical failure.

* Significant lysis.

simple sequence DNA. In any event the presence of DNA sequences of 90% G+C is likely to enhance pairing of homologous sequences because G+C duplexes are very stable (so that a shorter duplex is required for the minimal stability to hold a newly nucleated hybrid) and as essentially only two bases are involved the complexity of this DNA is very low and hence the probability of finding a 'homologous' sequence is high.

These arguments do not explain why the frequency of a number of different alleles of the wild mouse or human popula-

tion *H-2* or HLA is high compared with other gene systems. This is best explained by invoking a selective advantage to the individual with a new allele, for example, in cytotoxic T-cell mediated resistance to viral infection but is beyond the scope of this article.

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Location and primary structure of a major antigenic site for poliovirus neutralization

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We have determined a major antigenic site for virus neutralization on the capsid protein VP1 of poliovirus type 3. Antigenic mutant viruses selected for resistance to individual monoclonal antibodies had point mutations concentrated in a region 277-294 bases downstream from the start of the region of viral RNA coding for VP1. These findings provide the basis for an improved understanding of the molecular basis of virus neutralization.

THE causative agents of poliomyelitis¹, classified into poliovirus types 1, 2 and 3 on the basis of their serological characteristics, are members of the family Picornaviridae, a large group of viruses of considerable importance in human and veterinary disease. They are small viruses² possessing single-stranded, messenger-sense RNA genomes of about 2.6×10^6 molecular weight (~ 7.5 kilobases) enclosed in a protein capsid composed of 60 copies each of four virus-encoded polypeptides VP1, VP2, VP3 and VP4, ranging in molecular weight from 4,000 to 30,000. Specific antibodies capable of neutralizing virus infectivity are considered to be the major mechanism of protection against infection and disease. However, information on the nature of the antigenic structures involved in poliovirus neutralization is incomplete and the precise mechanisms by which virus is neutralized by antibody remain uncertain.

Here we describe the analysis of the antigenic structure of poliovirus type 3 by the isolation and characterization of mutant viruses resistant to neutralizing monoclonal antibodies. A major antigenic site involved in the neutralization of type 3 poliovirus has been identified in this way.

Isolation of mutant viruses

Four preparations of well-characterized, closely related type 3 polioviruses were used in the study. P3-Leon-USA-1947, the virulent precursor of the live attenuated Sabin type 3 vaccine strain³ was obtained from Dr A. Sabin. Most of the work described here concerned a plaque-purified isolate derived from

this preparation, designated Leon 970. The live attenuated Sabin type 3 vaccine strain (WHO vaccine reference preparation) and a plaque-purified derivative of it, designated Sabin 411, were also used. These viruses are also the subject of cloning and sequencing studies in our laboratories.

The preparation of rat and mouse hybridoma cell lines secreting monoclonal antibodies to poliovirus type 3 is described elsewhere (refs 4, 5 and M.F., in preparation).

A total of 103 monoclonal antibody-resistant plaques were picked in independent selections from Leon 970 using six different monoclonal antibodies and the virus eluted from each plaque was subjected to a second cycle of selection. Of the 103 primary plaques, 101 were *bona fide* mutants as their titres were unaffected by the presence of the selecting antibody in the second cycle of selection.

The frequencies with which mutants were isolated from the parental virus populations are expressed in Table 1 as the number of parental virus plaques per mutant for P3-Leon-USA-1947, Leon 970, Sabin type 3 vaccine, and Sabin 411. Plaque purification of parental virus did not affect the frequency of antigenic mutants obtained for either P3-Leon-USA-1947 or the Sabin type 3 vaccine strain. This agrees with present views on the nature of the virus RNA genome which is regarded as highly mutable, rapidly achieving an equilibrium level of heterogeneity on passage⁶.

Antigenic mutants could be isolated from P3-Leon-USA-1947 at 10 times the frequency with which they were obtained

Table 1 Frequency of mutant type 3 polioviruses resistant to monoclonal antibodies

Virus	Monoclonal antibody					
	25-1-14	25-4-12	25-5-5	27-4-4	132	134
P3-Leon-USA-1947	3.1	3.1	2.9	3.8	—	—
P3-Leon-USA-1947 clone 970	3.1	3.2	3.1	3.9	3.9	4.0
P3-Sabin vaccine	4.4	4.4	4.5	5.0	—	—
Sabin type 3 vaccine clone 411	4.5	4.3	4.3	4.8	—	—

Virus was incubated overnight at 4 °C with a concentration of monoclonal antibody at least 100-fold greater than its end point titre in tissue culture 50% infective dose (TCID₅₀) neutralization tests, and an appropriate dilution of this virus seeded onto confluent monolayers of HEP2c cells. Unadsorbed virus was washed off after 30 min and the cells overlaid with 1.2% agar in L15 medium containing monoclonal antibody at one-tenth the concentration of that of the original incubation mixture. Cell sheets were stained after 3 days at 35 °C with an overlay of 0.1% neutral red in 1.2% agar and plaques picked (primary plaques). Variants of Leon 970 isolated from these plaques were subjected to a second cycle of selection to confirm that they were truly resistant. Frequencies are expressed as the titre of parental type virus (as log₁₀) per mutant. The virus neutralization titres of the six monoclonal antibodies when challenged with 100 TCID₅₀ of P3-Leon-USA-1947 were 1:400 (25-1-14), 1:1,600 (25-4-12), 1:200 (25-5-5), >1:10,000 (27-4-4), 1:640 (132) and >1:10,000 (134).

Table 2 Pattern of resistance of mutants of type 3 poliovirus selected with neutralizing antibodies

Mutant group	Monoclonal antibody																No. of isolates	Selecting monoclonal antibodies	
	a								b										
	25-1-14	25-4-12	25-5-5	132	165	175	134	27-4-4	113	197	194	204	208	214	179	199			
1	r	r	r					r		r				r	r	r	38	25-1-14	25-5-5
2	r	r	r	r	r					r	r			r	r	r	15	25-4-12	27-4-4
3	r	r	r	r	r	r		r	r	r	r	r	r		r	r	2	25-1-14	25-5-5
4	r	r	r	r	r			r	r	r	r	r	r	r	r	r	9	25-4-12	132
5				r	r	r	r	r	r	r	r		r	r	r	r	9	25-5-5	27-4-4
6				r	r		r	r	r		r		r	r	r	r	3	132	
7				r	r	r	r	r	r		r	r	r	r	r	r	2	134	
8							r	r	r	r			r	r			9	27-4-4	
9	r	r	r	r	r	r	r	r	r	r	r		r	r	r	r	2	27-4-4	134
10	r		r	r	r	r	r	r	r	r	r	r	r	r	r	r	4	132	

a, Virus eluted from the agar plugs of the secondary plaques was seeded onto confluent Hep2c cell monolayers in 24-well tissue culture plates. Minimal essential medium was then added containing either no monoclonal antibody, the selecting monoclonal antibody or another monoclonal antibody of the panel to be studied. Wells were examined daily until the control well showed a cytopathic effect and then scored for the presence or absence of a cytopathic effect. All isolates were examined in this way. b, A representative strain was selected from each mutant group and assayed for sensitivity to the monoclonal antibody panel by reduction in plaque number. Virus was treated with an appropriate antibody dilution overnight at 4 °C then assayed for infectious virus by plaque assay as described in Table 1. Virus was considered to be resistant if the dilution of antibody required to reduce the number of plaques to 50% of the control was less than 1:10, and sensitive if this dilution was greater than 1:160. r, Resistant.

from the Sabin type 3 vaccine. This implies that the Sabin type 3 vaccine strain is genetically more stable in culture than the Leon virus from which it was derived. The frequencies with which antigenic mutants were obtained from the Sabin type 3 vaccine strain were comparable to those reported for influenza⁷, rabies⁸ and parainfluenza⁹ viruses and ranged from 1 in 10⁴ to 1 in 10⁵ depending on the monoclonal antibody used.

Antigenic characterization of mutant viruses

It is assumed that an antigenic site can be composed of several distinct epitopes each of which is specifically recognized by a different antibody-binding site¹⁰. Here, two epitopes are said to be in the same operationally defined antigenic site if a mutation affecting the reactions of one epitope frequently affects the reactions of the second or if they can both be linked in this way to a third different epitope¹⁰. An antigenic site defined by these operational criteria may correspond to a physicochemical feature of the antigen.

The doubly plaque-purified mutants isolated from Leon 970 were examined to determine how many different independent operationally defined antigenic sites were recognized by the 16 monoclonal antibodies used in the study.

All 101 isolates were examined for neutralization by eight of the monoclonal antibodies listed in Table 2. As eight of the

isolates were of insufficiently high titre for unambiguous patterns of reaction to be established, they were not studied further. The remaining 93 mutants could be classified into 10 groups on the basis of their neutralization patterns. Representatives of each group were then tested for neutralization by eight additional monoclonal antibodies. Table 2 shows the reaction patterns, the number of isolates assigned to each group and the antibodies used to select them.

The data shown in Table 2 provide strong evidence that all the epitopes recognized by the 16 monoclonal antibodies used in this study are interdependent and clustered into a single operationally defined antigenic site. This conclusion is based on the observation that all mutations to a resistant phenotype obtained conferred resistance to at least 8 of the 16 monoclonal antibodies tested (group 1). Members of some groups (9, 10) were resistant to 15 of the 16 antibodies and there were mutant groups which showed resistance to all possible pairs of monoclonal antibodies. Resistance to 134 and 25-4-12 co-varied in only one mutant group (group 10). However, resistance to both 134 and 25-4-12 co-varied with resistance to 132 for at least five of the mutant groups. The data in Table 2 reveal 11 distinct patterns of reaction of the panel of monoclonal antibodies with the 10 mutant groups. At least 11 different epitopes are therefore recognized by the 16 antibodies. In addition, the epitopes

recognized by 132 and 165 are distinct as these antibodies differ in their specificity for different strains², and 165 inhibits binding of radiolabelled virus to cells at concentrations which neutralize infectivity whereas 132 does not (P.D.M., unpublished). Similarly, 25-1-14 prevents adsorption of virus to cells at neutralizing concentrations, whereas 25-5-5 does not. These findings suggest that the panel of monoclonal antibodies distinguishes at least 13 different epitopes. Several of these different epitopes have previously been distinguished by the strain specificity of the monoclonal antibodies^{4,5} and their specificity for sub-viral components (M.F., in preparation).

The patterns of reactions of representative poliovirus strains from each group in antigen-blocking single radial-immunodiffusion tests were the same as in neutralization tests, suggesting that the mutations conferred resistance directly, by affecting binding of antibody to the virions.

All mutants were recognizable as type 3 polioviruses as they were all neutralized by type-specific polyclonal sera (data not shown) and by at least one individual monoclonal antibody. The mutations they possess are therefore not expected to have a profound effect on their overall immunogenicity.

Biochemical characterization of mutant viruses

The pattern given by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the virus-encoded proteins synthesized in cells infected with poliovirus is often characteristic of the infecting poliovirus strain¹¹⁻¹³ and was used to examine all 101 mutants from Leon 970. Typical autoradiograms are shown in Fig. 1. Most mutants gave a pattern of proteins indistinguishable from that induced by the parental strain (Fig. 1A). However, all nine mutants which had been assigned to mutant group 5 on the basis of their antigenic properties possessed a VP1 having a greatly increased mobility (Fig. 1B), while all four mutants classified into mutant group 10 showed a smaller but highly reproducible migrational change in this protein (Fig. 1C). No migrational changes were observed in any other virus-specified polypeptide for any mutant examined. These findings are consistent with the classification of the viruses into their specific mutant groups on the basis of their patterns of neutralization and suggest that at least for groups 5 and 10 the resistance to specific monoclonal antibodies involved mutational changes affecting VP1.

T1 RNase oligonucleotide mapping of mutant strains

T1 RNase oligonucleotide mapping of poliovirus RNA provides a more sensitive means of strain characterization than SDS-PAGE of infected cell proteins¹¹⁻¹⁵. The larger characteristic oligonucleotides of the map produced from Sabin type 3 virus RNA have been numbered for convenience of analysis¹⁵, and as the same oligonucleotides are also found in maps of P3-Leon-USA-1947 (P.D.M., unpublished), the same numbering system has been used here. The oligonucleotides of specific interest in the present studies are identified in the T1 RNase oligonucleotide map of RNA from the parental strain Leon 970 presented in Fig. 2a.

Characteristic changes involving the loss of oligonucleotide 31 were found in maps of 6 of the 10 mutant groups (open circles on the left side of the autoradiograms in Fig. 2b-f, j, k). This was shown to be the case for all isolates of these groups examined. The absence of oligonucleotide 31 from maps of the RNA of these mutants was confirmed by preparing maps from mixtures of T1 RNase digests of RNA from Leon 970 and similar digests of RNA from mutant viruses (data not shown).

In addition, maps of five of these six mutant groups contained an additional oligonucleotide, different for each group, but found in maps of all mutants of the group examined (bold arrows on the left-hand side of the autoradiograms in Fig. 2b, d-f, j, k). The migration rates of these oligonucleotides were consistent with the view that they were derived from oligonucleotide 31 by single point mutations. Thus the position of the novel oligonucleotides in maps of three of the mutant groups suggested a displacement of oligonucleotide 31 in the first dimension of electrophoresis, in which separation is on the basis of net nucleotide composition, implying C→U (group 1), A→U (group 8) or C→A (group 3) mutation^{16,17} while the novel oligonucleotides of two other mutant groups suggested a displacement in the second dimension in which separation is on the basis of size, consistent with the loss (group 4) or gain (group 9) of a G residue resulting in an alteration in the size of the oligonucleotide. The complete absence of a novel oligonucleotide in the vicinity of oligonucleotide 31 in maps of group 2 mutants implies the insertion of a central G residue, resulting in cleavage to smaller undetected oligonucleotides.

Maps of several strains from each group were examined. Both the absence of oligonucleotide 31 and the presence, where

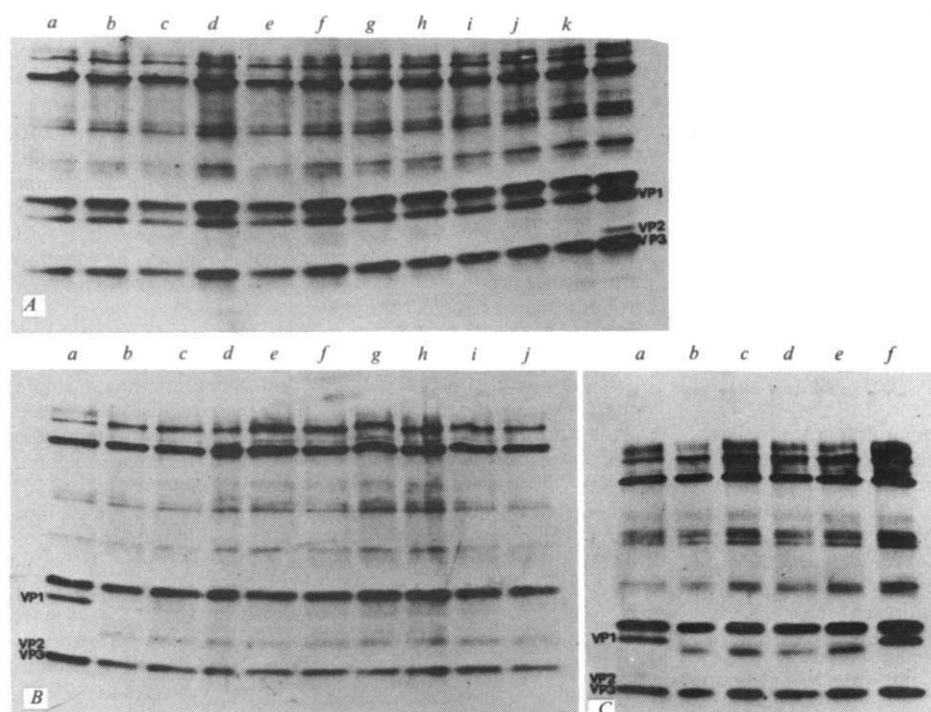


Fig. 1 Autoradiograms of the radiolabelled proteins produced by infection of HEP2c cells with virus derived from independent primary plaques. **A:** a-k, cells infected with group 1 mutants; l, cells infected with parental virus 970. **B:** a, cells infected with parental virus; b-j, cells infected with group 5 mutants. **C:** a, f, cells infected with parental virus; b-e, cells infected with group 10 mutants.

Methods: working pools were grown of all 93 mutant viruses using as a seed the harvest from the FB24 well containing the original selecting antibody in the testing system described in Table 2. HEP2c cells were infected with 0.1 ml of these working pools, incubated at 35 °C and the pattern of proteins synthesized examined by pulsing the cells with ³⁵S-methionine at 6-7 h after infection and resolving the radiolabelled proteins on a SDS (10-30%)-polyacrylamide linear gradient gel¹⁵.

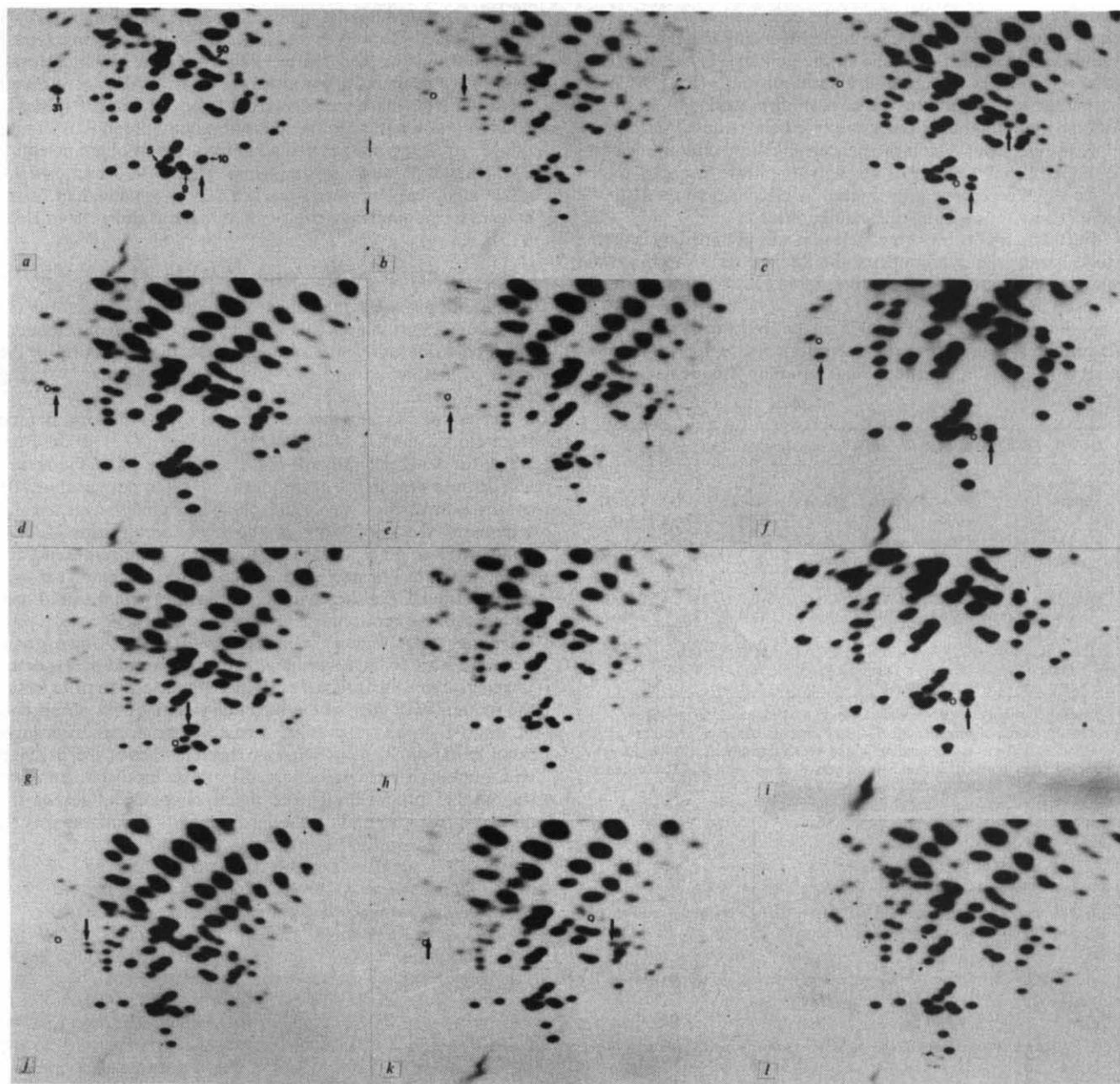


Fig. 2 T1 RNase oligonucleotide maps of RNA extracted from purified viruses representative of the parental and mutant groups grown in HEP2c cells in the presence of inorganic phosphate¹⁹: *a*, 970; *b*, group 1; *c*, group 2; *d*, group 3; *e*, group 4; *f*, group 4; *g*, group 5; *h*, group 6; *i*, group 7; *j*, group 8; *k*, group 9; *l*, group 10. The absence of parental spots is indicated by open circles. The arrow in *a* indicates shadow spot 9A; arrows in other cases indicate spots not found in the parent.

appropriate, of a novel oligonucleotide possibly derived from it, were highly characteristic of the maps of strains of a particular group and correlated with the antigenic properties of the mutants. However, there were other alterations in maps of some of the isolates. An additional oligonucleotide was found in maps of the RNA of the group 2 mutant shown in Fig. 2c (indicated by a bold arrow on the right of the autoradiogram). As this was the case for only one of three mutants for which maps were prepared, it is unlikely to be associated with the antigenic properties of the mutants.

In maps of some mutants, oligonucleotide 9 was absent and a novel oligonucleotide, migrating just below oligonucleotide 10 (bold arrow at the centre of Fig. 2c, *f*, *i*) was present. A faint oligonucleotide was consistently visible in this position in maps of the RNA from Leon 970 (bold arrow in Fig. 2a) suggesting that the parental virus was heterogeneous and that mutants were derived from one or other of the two principal

sub-populations. Maps of two mutants from group 4 had the parental oligonucleotide 9, while the map of a third had the mutant oligonucleotide (Fig. 2e, *f*). All three were the same in their reactions with monoclonal antibodies and all had maps lacking oligonucleotide 31, but with the characteristic oligonucleotide of group 4 mutants. Antigenic reactivity of this group was therefore unaffected by the absence of oligonucleotide 9 and it is thought to be unlikely that it affects the antigenic properties of other groups. The additional modifications of the maps of group 9 mutants indicated in Fig. 2k were found in maps of both isolates obtained.

A different type of characteristic change was observed in maps of RNA of group 5 mutants, in which oligonucleotide 5 was absent and a smaller oligonucleotide was present (Fig. 2g). This alteration was observed in all members of this group. As these mutants were also known to have an abnormal virion protein, VP1 (Fig. 1B), it was expected that oligonucleotide 5

A + C-rich species of intermediate length, consistent with the composition of the 16-base sequence identified in the parental strain (Table 3).

The location of oligonucleotide 31 in the region of the poliovirus 3 genome coding for VP1, implies that VP1 contains the major antigenic site involved in virus neutralization detected in these studies. This is consistent with the migrational changes of VP1 observed for the mutant viruses of groups 5 and 10. The coding assignments in the only open reading frame in the region of oligonucleotide 31 are shown in Table 3. The three mutant oligonucleotides sequenced all showed mutations affecting coding function, and the predicted substitutions were not associated with extreme changes in charge or hydrophobicity and occurred at three consecutive positions in the sequence. This may suggest that there are severe restrictions on the type of mutation compatible with virus growth and viability.

Conclusions

The 16 neutralizing monoclonal antibodies specific for type 3 poliovirus used in this study were of widely varied origins and specificity but appeared to recognize only one operationally defined antigenic site. Other workers have reported the existence of two antigenic sites in poliovirus type 1 based on the isolation of populations resistant to one of two monoclonal antibodies²³. While it is possible that type 1 poliovirus has multiple, readily detectable, independent antigenic sites, it is also possible that more extensive studies involving a larger panel of monoclonal antibodies would link the two neutralizing epitopes described. The findings reported here do not, however, exclude the possibility that other less easily identified independently mutable antigenic sites exist in type 3 poliovirus. For foot-and-mouth disease virus, another picornavirus, two distinct fragments of VP1²⁴ or synthetic peptides corresponding to these areas²⁵ have been shown to induce neutralizing antibody. This suggests either that there are two antigenic sites, or that these sequences are brought together to form a single site by the three-dimensional structure of the protein. Multiple independent sites for neutralization have been reported in similar studies of the surface glycoproteins of influenza²⁶, rabies⁸ and parainfluenza viruses⁹. In the case of influenza haemagglutinin it was shown that the mutations which were operationally clustered within a single independent or semi-independent antigenic site were also clustered in the three-dimensional structure of the molecule²⁷, and that there were four such clusters corresponding to the four independent sites. The molecular characterization of the poliovirus mutants suggests that several of the epitopes of the antigenic site detected here are also physically clustered into a small area of a virus protein (VP1). However, it is also possible that some of the other mutations are distant from oligonucleotide 31 and yet

affect the presentation of an epitope within this antigenic site, by virtue of a structural interdependence of the capsid proteins of the poliovirus particle.

The antigenic site for neutralization of poliovirus defined here seems to be located within VP1. This is consistent with the findings reported for other picornaviruses such as foot-and-mouth disease virus^{28,29} and Mengo virus³⁰ for which VP1 has been shown to be capable of inducing a weak neutralizing antibody response. It is also consistent with the known exposed location of VP1 on poliovirus³¹, its immunodominance³² and recent studies involving the cross-linking of specific antibodies to poliovirus²³. It has been reported that poliovirus VP1 will induce neutralizing antibodies³³. On the other hand, this has also been described for preparations of the other capsid proteins¹ and neutralizing antibody can be induced with purified preparations of VP2 of Cocksackie virus³⁴, another picornavirus, so that the existence of antigenic sites on other proteins cannot be excluded.

For several of the mutant groups from both Leon 970 and the Sabin type 3 vaccine strain, the pattern of antigenic reaction appears to be associated with characteristic mutations of the T1 RNase-resistant oligonucleotide 31. This oligonucleotide has been unchanged in any of the isolates obtained from recipients of Sabin type 3 vaccine examined so far^{11,14,15} or in several laboratory plaque-purified isolates of Leon virus (data not shown), although these show modifications in other oligonucleotides. It is therefore unlikely that it constitutes a mutational hotspot. If the amino acid sequence which this oligonucleotide encodes is either crucial to the presentation of the antigenic site or more probably represents at least part of the antigenic site itself, it would be expected to be poorly conserved between strains which can be distinguished serologically such as type 1 and type 3. This is in fact the case (Fig. 3), although the sequences adjacent to the region are well conserved. The sequence encoded by oligonucleotide 31 is therefore a good candidate for a peptide inducing a neutralizing immune response to poliovirus. Immunogenic peptides have been described for other viruses^{25,35-37} and have been shown to stimulate antibodies reacting with native virions.

While the region of the genome homologous to oligonucleotide 31 shows considerable variation between serotypes, an even greater variation is observed between poliovirus types 1 and 3 in the region coding for the N-terminus of VP1 (see Fig. 3). Our studies provide no evidence that this hypervariable region is directly involved in the neutralization of the virus.

Finally the fact that oligonucleotide 31 has been invariably found in maps of the RNA of all Sabin type 3 vaccine-related viruses isolated after human passage^{11,14,15}, suggests that the immune response plays little part in the generation of the biological diversity of such isolates found in nature.

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Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences

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At least one of the HeLa cell factors necessary for correct in vitro transcription from conalbumin or adenovirus late promoters binds to the region of the template that contains the 'TATA' box. This binding, which occurs in the absence of RNA polymerase B, results in the formation of stable preinitiation complexes.

ALTHOUGH gene expression in eukaryotes can be regulated at several different levels, initiation of transcription by RNA polymerase B is undoubtedly a key step in the control of mRNA synthesis from many cellular and viral genes (refs 1–3 and references therein). The characterization of the basic components and events responsible for initiation of transcription is essential if we are to understand the molecular mechanisms by which this step is controlled by specific regulatory signals. In prokaryotes, such studies have been greatly facilitated by the availability of simple *in vitro* systems in which purified, well defined DNA templates are accurately transcribed by purified RNA polymerase. In contrast, purified eukaryotic RNA polymerase B cannot accurately initiate transcription *in vitro* on purified templates unless the system is supplemented with crude cellular extracts^{4,5}. These crude systems have been extensively and successfully used to analyse the promoter DNA sequences required for 'accurate' transcription by RNA polymerase B (see refs 6–9 for reviews; 'accurate transcription' refers here to initiation at the specific *in vivo* start sites (cap sites)). For instance, it has been conclusively demonstrated that, for those genes which possess it, the TATA box sequence is essential for accurate initiation of transcription (see refs 6–10 for reviews).

Chromatography of crude extracts gives multiple partially purified fractions, all of which are required to restore accurate transcription^{8,11}. The active components of these fractions must be highly conserved, as they are neither species nor tissue specific; they can direct specific initiation of transcription on a variety of heterologous protein-coding genes in the presence of RNA polymerase B obtained from various sources^{6–10}. However, it is not known how many of these fractions contain *bona fide* transcription factors actually involved in the specific initiation process, nor their mechanisms of action.

In the present study we have developed a procedure for the detection of sequence-specific DNA-binding initiation factor(s) present in a preparation partially purified from a crude extract. We report the existence in such a preparation of component(s) which bind specifically to the TATA box sequence and are indispensable for accurate *in vitro* initiation of transcription.

Partial purification of a HeLa cell extract promoting accurate transcription

A partially purified RNA polymerase B transcription factor preparation was obtained by chromatography of a HeLa cell S100 extract⁴ on heparin-Ultrogel (Fig. 1A). The leading edge of the main 0.6 M KCl peak—here called the H0.6 fraction—contained the factors necessary for accurate *in vitro* initiation of transcription at the adenovirus-2 (Ad2) major late promoter by RNA polymerase B (Fig. 1B, lanes 6–8) and on promoter-containing fragments of the conalbumin (see below), ovalbumin and SV40 early genes (B.L.D., unpublished observations). On

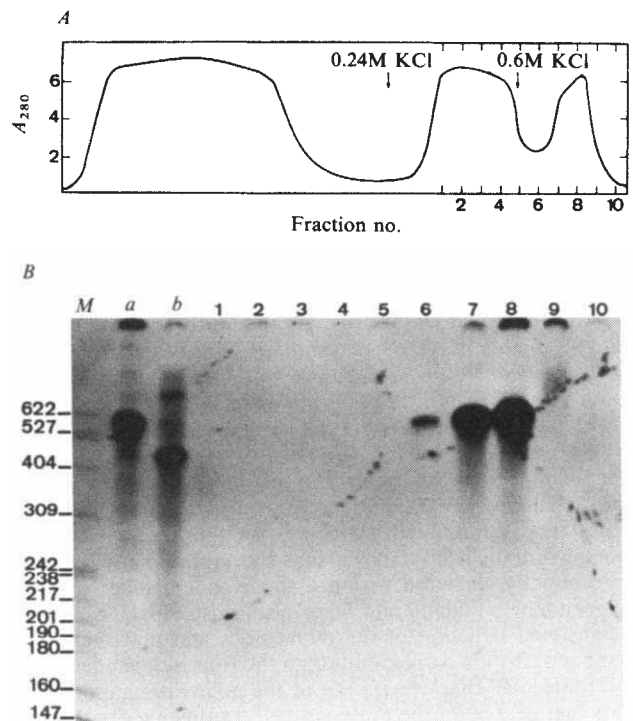


Fig. 1 Chromatography of a HeLa cell S100 extract on heparin-Ultrogel. **A**, A crude, dialysed S100 extract (50 ml) was prepared from 7×10^9 HeLa cells⁴ and applied to a column (2.5×10 cm) of heparin-Ultrogel (IBF) equilibrated with 50 mM Tris-HCl (pH 7.9), 100 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol and 20% glycerol (buffer A). The column was washed with 2 column volumes of buffer A, 1 column volume of buffer A containing 0.24 M KCl and finally, 1.5 column volumes of buffer A containing 0.6 M KCl. After elution of the flow-through peak, 4-ml fractions, 1–10, were collected. **B**, Fractions were dialysed against buffer A for 4 h at 4 °C and each fraction assayed for specific transcription activity (60 min at 25 °C) in 26- μ l (final volume) reaction mixtures containing 10 mM Tris-HCl (pH 7.9), 7.5 mM MgCl₂, 50 mM KCl, 8% glycerol, 0.2 mM dithiothreitol, 500 μ M each of ATP, UTP, GTP, 20 μ M [α -³²P]CTP (7,000 c.p.m. pmol⁻¹), 0.008 units of calf thymus RNA polymerase B²⁵ and 150 ng of the adenovirus-2 *TaqI* DNA fragment^{10,14}. After synthesis, RNA was purified and analysed on a 5% acrylamide-urea gel essentially as described previously¹⁵. Lanes *a* and *b*, 12 μ l of S100 and heparin flow-through, respectively; lanes 1–5, 10 μ l of 0.24 M KCl fractions 1–5, respectively; lanes 6–10, 10 μ l of 0.6 M KCl fractions 6–10, respectively; lane *M*, *MspI*-digested pBR322, 5' end-labelled with ³²P. The H0.6 fractions were quick-frozen in liquid nitrogen and stored at –80 °C.

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a cell basis the activity of the H0.6 fraction in promoting accurate transcription was at least equal to that of the corresponding S100 extract, whereas >90% of the protein was removed during this chromatographic step. Occasionally, addition of the flow-through fraction resulted in stimulation of specific transcription in assays containing the H0.6 fraction. About 30% of the endogenous RNA polymerase B was eluted in factor-containing (H0.6) fractions, as determined by titration with ^3H -amanitin¹². This corresponds to ~ 0.020 pmol of RNA polymerase B per $6\ \mu\text{l}$ of H0.6 (result not shown).

Tight binding of transcription factor to DNA

To test the possibility that a sequence-specific DNA-binding factor could be, at least in part, responsible for accurate transcription, we developed an assay which involves the sequential addition of two different DNA templates to incubation mixtures containing the H0.6 fraction (because the number of components actually necessary for accurate transcription is unknown, the term transcription factor is used here for simplicity). This assay, referred to as the 'prebinding' assay, was based on the assumption that, in conditions preventing transcription initiation (that is, in the absence of nucleoside triphosphates, NTPs), an initiation factor could bind more tightly to DNA fragments containing known promoter sequences than to fragments not containing such sequences. Accurate transcription of a second, distinguishable, template added to the reaction mixture 10 min later, but before initiation of transcription by NTP addition, would then be strongly reduced or even eliminated, provided that the first template were present in excess and its interaction with the factor sufficiently stable. Absence, in the first DNA, of sequences necessary for the specific binding of the factor would then be revealed by efficient and accurate transcription of the second template.

Before the prebinding assay, the H0.6 fraction was incubated with poly(I)·poly(C) (at a concentration determined previously by titration) to adsorb nonspecific DNA-binding proteins. Addition of poly(I)·poly(C) reduces the amount of promoter-containing DNA template required to detect specific transcription and improves the efficiency of the assay in discriminating between sequence-specific and nonspecific DNA-factor interactions (see below and data not shown).

When the promoter sequence-containing 4-kilobase (kb) conalbumin *EcoRI*'b' fragment¹³ and the Ad2 major late transcription unit *TaqI* fragment^{10,14} were individually or simultaneously assayed in the presence of the H0.6 fraction, 'run-off' RNAs of 500 (ref. 15) and 560 (refs 10, 14) nucleotides, respectively, characteristic of accurate transcription, were produced (Fig. 2, lanes a-c). The results were drastically different when the DNA templates were added sequentially. Either the Ad2 (lanes f, g) or conalbumin (lanes d, e) template was incubated for 10 min at 25°C with the H0.6 fraction in the absence of NTPs. The second template was then added and incubation was continued at 25°C for 10 min, after which transcription was initiated by addition of NTPs and calf thymus RNA polymerase B (the latter was omitted in lanes e and g). After 15 min at 25°C the run-off RNA transcripts were analysed. The results indicate that the DNA added first (prebinding DNA) is essentially the only DNA specifically transcribed. This observation suggests that some component of the H0.6 fraction involved in the initiation of accurate transcription is stably associated with this DNA. (The term 'stable' is used here and below when referring to factor-DNA associations strong enough significantly to reduce accurate transcription from the second template added after the prebinding period.) The finding that addition of exogenous calf thymus RNA polymerase B, together with the NTPs, stimulated accurate transcription from the prebinding template, indicates that the number of DNA-factor complexes formed during the prebinding period exceeds the amount of endogenous polymerase present in the H0.6 fraction (compare Fig. 2, lanes d, e and f, g). The effect of the sequential addition of the DNA templates was independent of whether the exogenous polymerase was present during the prebinding

period and, for convenience, it was added to the prebinding mixture in all experiments described below except those of Fig. 6.

Using the prebinding conditions and DNA concentrations described in Fig. 2 legend, accurate transcription of the second template was decreased to about 5% of that seen with simultaneous DNA additions. With lower amounts of prebinding DNA (not shown, but see also Fig. 3), correspondingly higher levels of accurate transcription were seen from the second templates. On a weight basis, the 4-kb conalbumin 'b' fragment was about three times less efficient than the 0.8-kb Ad2 *TaqI* fragment in reducing accurate transcription from the second template. This result indicated that a significant fraction of the prebinding complexes involved either specific DNA sequences or DNA termini.

Specific binding of transcription factor to DNA fragments containing promoter sequences

The prebinding assay was used to compare the efficiencies of various subcloned regions of the conalbumin b fragment in forming stable complexes. Figure 3B shows that the 164-bp *AluI* fragment (−102 to +62, Fig. 3A), which contains the conalbumin promoter region^{10,15}, is 18-fold more efficient (on a weight basis) than the 4-kb *EcoRI* b fragment. This difference

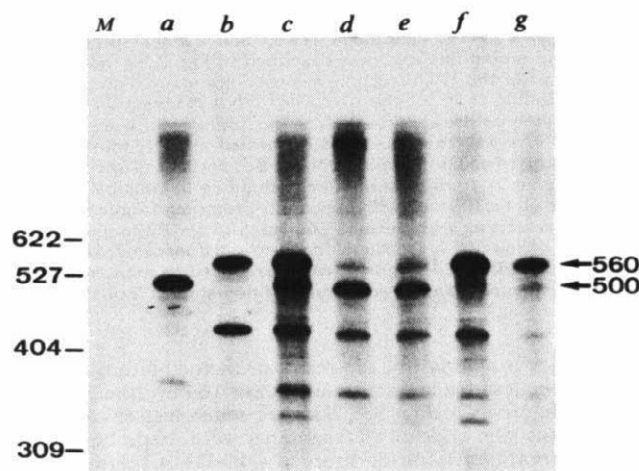
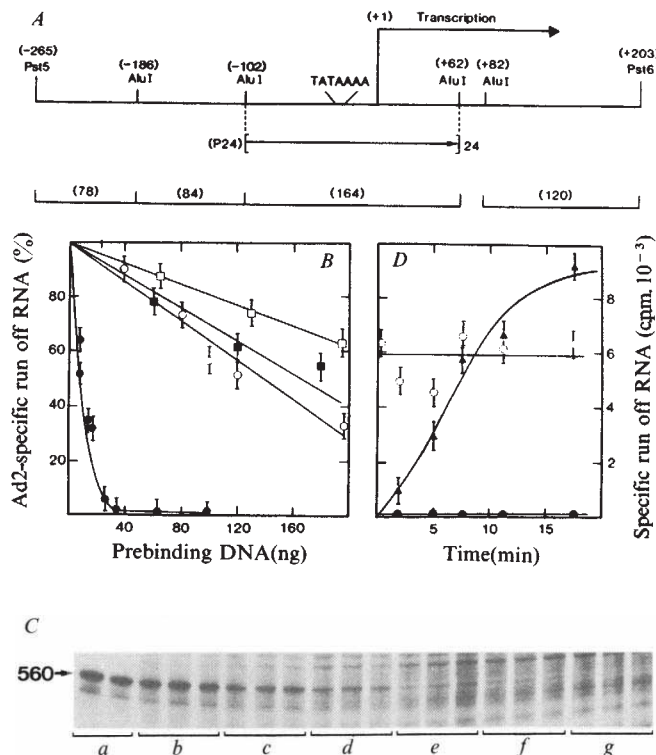


Fig. 2 The DNA prebinding assay. Lane a, prebinding with 210 ng of conalbumin 'b' DNA; b, prebinding with 70 ng of Ad2 *TaqI* DNA; c, simultaneous prebinding with 210 ng of conalbumin 'b' and 70 ng of Ad2 *TaqI* DNAs; d, prebinding with 210 ng of conalbumin 'b' DNA followed 10 min later by addition of 70 ng of Ad2 *TaqI* DNA; e, same as d except that no calf thymus RNA polymerase B was added to the assay; f, prebinding with 70 ng Ad2 *TaqI* DNA followed 10 min later by addition of 210 ng of conalbumin 'b' DNA; g, same as f except that no exogenous RNA polymerase B was added to the assay. The 430-nucleotide RNA band present in lanes b–g is a polymerase C product transcribed from the Ad2 template (not shown).

Methods: The H0.6 fraction (Fig. 1) ($6\ \mu\text{l}$) was preincubated at 25°C for 3 min with 125 ng of poly(I)·poly(C) (Miles) in 21- μl reaction mixtures containing the components listed in Fig. 1 legend except that no ribonucleoside triphosphates (NTPs) or calf thymus RNA polymerase B were present. Either the conalbumin 'b'¹³, Ad2 *TaqI* or both DNA fragments (see text) were then added, to initiate the prebinding reaction. After incubation for 10 min at 25°C, the second DNA template (either Ad2 *TaqI* or conalbumin 'b') was added and the incubation continued for 10 min before the initiation of transcription by the addition of NTPs and, unless otherwise stated, 0.008 units (0.06 pmol) of calf thymus RNA polymerase B. RNA was synthesized in 26- μl (final volume) reactions for 15 min (25°C) and analysed as described in Fig. 1 legend.

Fig. 3 Demonstration of sequence-specific DNA binding of RNA polymerase B transcription factor(s). **A**, Map of the promoter-containing 468-bp conalbumin *Pst*5-6 fragment¹³ showing the positions, in terms of base pairs relative to the cap site (+1), of *Alu*I cleavage. The numbers in parentheses on the lower line indicate the size (bp) of the associated DNA fragments. **B**, Increasing amounts of various purified DNA fragments, corresponding to regions near the conalbumin mRNA cap site (see **A**), were added as first (prebinding) DNA in prebinding assays and incubated as described in the legend to Fig. 2 (6 μ l of H0.6 per assay). The amount of specific transcription of the second DNA template (Ad2 *Taq*I fragment) was then quantified by counting gel slices and/or integrating densitometer scans of autoradiograms. The per cent of Ad2 *Taq*I-specific transcription relative to assays containing no prebinding DNA is plotted as a function of the prebinding DNA concentration. Prebinding DNA was: $\circ$ —, the 4-kb conalbumin 'b' fragment; $\bullet$ —, the 164-bp (–102 to +62) *Alu*I fragment or the 4-kb 'P24' fragment consisting of 24 tandem head-to-tail repeats of the 164-bp *Alu*I region (see **A** and below); $\square$ —, the 78-bp (–265 to –186) *Pst*I-*Alu*I or 84-bp (–186 to –102) *Alu*I fragments; $\blacksquare$ —, the 120-bp (+82 to +203) *Alu*I-*Pst*I fragment. In this, as well as in all the prebinding assays described below and in the other figures, the data points represent the average of at least triplicate assays at each DNA concentration. The bars indicate the range of triplicate determinations. **C**, A typical autoradiogram of a prebinding assay, with the P24 fragment as the first DNA, followed by addition of the Ad2 *Taq*I fragment which yields the 560-nucleotide run-off RNA. The reactions shown in lanes a–g contained 0, 8.5, 17, 26.5, 34, 68 and 102 ng of P24 DNA, respectively. **D**, Correlation between specific and 'slowly dissociating' factor-DNA complexes. The conalbumin 'b' (160 ng) or P24 (170 ng) fragments were added as first (prebinding) DNAs in prebinding assays as described in Fig. 2 legend, except that 10 μ l of the H0.6 fraction was used per assay. After 10 min of prebinding at 25 °C, the Ad2 *Taq*I DNA fragment (140 ng) was added and the incubations were continued for increasing time intervals, as indicated on the abscissa, before the initiation of transcription by NTP addition. Specific run-off transcripts from conalbumin 'b' and Ad2 *Taq*I DNAs were analysed and quantified after 15 min of transcription at 25 °C. $\blacktriangle$ —, Amount of specific Ad2 *Taq*I transcription after prebinding to conalbumin 'b' DNA; $\circ$ —, amount of specific conalbumin 'b' transcription after prebinding to conalbumin 'b' DNA. All DNA concentrations were determined by the DABA method²⁶. Cloning of the P24 conalbumin multi-promoter fragment: the P24 fragment was constructed in three successive self-ligation (T4 ligase) reactions starting with purified 164-bp *Alu*I (–102 to +62) DNA; these reactions were interrupted by intermediate cloning steps involving ligation of head-to-tail tetramer, head-to-tail dodecamer and head-to-tail 24-mer fragments into the *Bam*HI, *Eco*RI and *Cla*I sites, respectively, of pBR322; the P24 fragment can be reduced to monomers by digestion with *Bam*HI and *Alu*I and its structure has also been verified by DNA sequencing and *in vitro* transcription studies (not shown). For all fragments, DNA termini were made blunt by *S*₁ nuclease treatment before testing.



in efficiency is clearly not the result of factor binding to DNA termini, because fragments smaller than 164 bp (the 78, 84 or 120 bp fragments in Fig. 3A, B) were much less efficient on a weight basis (the ends of all fragments were made blunt by *S*₁ nuclease treatment). Furthermore, a 4-kb DNA fragment (P24 in Fig. 3A–C) consisting of 24 tandem head-to-tail repeats of the 164-bp *Alu*I fragment was as efficient as the 164-bp monomer in reducing accurate transcription from the second template. The striking efficiency of the 164-bp promoter-containing sequence was also not due to differences in the rates of formation of DNA-factor complexes on the various prebinding DNAs, because variation in the time of prebinding from 4 to 25 min did not change the relative efficiencies shown in Fig. 3B (J.M.E., unpublished results). In illustration of the rational stated above for preincubation of the H0.6 fraction with poly(I)·poly(C), we note here that in the absence of this step there was only a fivefold difference in complexation efficiency between the P24 and conalbumin *Eco*RI b fragments (not shown).

Figure 3B, C and D show that the complexes formed between the transcription factor and the 164-bp *Alu*I (or P24) fragment do not dissociate during the assay. (The absence of Ad2 *Taq*I specific run-off transcription after prebinding to P24 DNA does not simply reflect some nonspecific inhibition of transcription by concentrated promoter sequence, because efficient Ad2- and P24-specific transcription was detected when P24 and Ad2 DNAs were simultaneously added to prebinding assays (not shown).) In contrast, when using the 4-kb conalbumin *Eco*RI b fragment as the prebinding DNA, the increase in incubation time after addition of the Ad2 *Taq*I template (but before NTP

addition), led to a progressive increase in accurate transcription from the Ad2 major late promoter (Fig. 3D). However, there was no concomitant decrease in accurate transcription from the conalbumin promoter. These observations suggest that at least two types of complex are formed during the prebinding incubation period with the conalbumin b fragment. One type, functionally 'committed' to specific transcriptional events on the prebinding template, seems to be as stable as the complexes formed on P24 DNA, whereas the other, presumably bound at nonspecific sites, dissociates much more easily from the prebinding DNA and becomes functionally associated with the Ad2 template.

The formation of nonspecific, less stable factor-DNA complexes probably accounts for the apparent inhibition by DNA fragments which do not contain promoter sequences. The precise nature of these nonspecific complexes is unknown and parameters, such as fragment size or base composition, which might affect the kinetics of factor redistribution onto the second DNA, have not been characterized. Therefore, we have not emphasized in the figures the apparent differences in efficiency between such fragments and have considered them, for the purposes of discussion, to be more or less equivalent. Only the complexes on fragments which demonstrate factor-binding efficiencies comparable to the conalbumin 164-bp fragment are referred to as specific. Theoretically, inhibition in the prebinding assay by the multi-promoter P24 fragment should be 24 times more efficient than by the conalbumin b fragment. The lower value of 18-fold seen in Fig. 3B is probably at least partly explained by the incomplete removal of nonspecifically bound factor from the prebinding conalbumin b fragment.

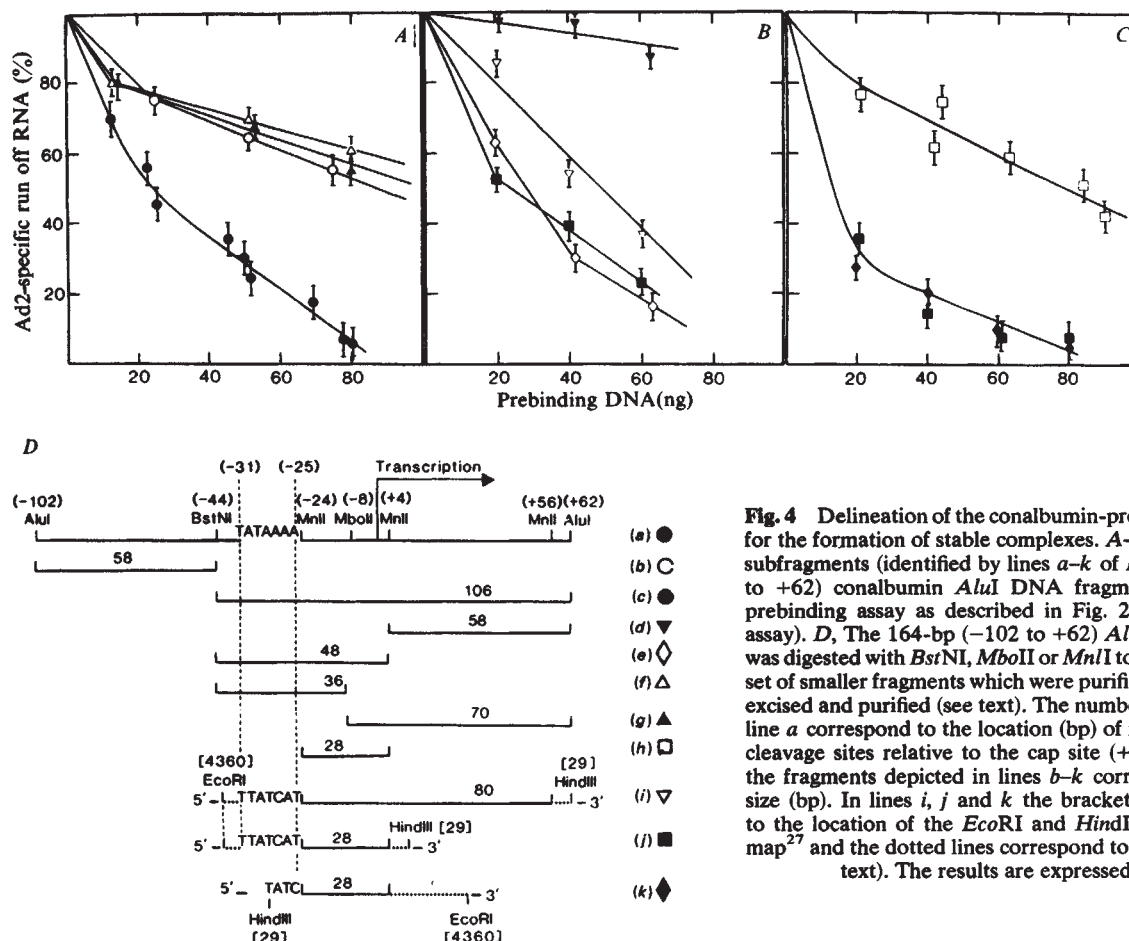


Fig. 4 Delineation of the conalbumin-promoter region important for the formation of stable complexes. *A-C*, Various blunt-ended subfragments (identified by lines *a-k* of *D*) of the 164-bp (–102 to +62) conalbumin *AluI* DNA fragment were tested in the prebinding assay as described in Fig. 2 legend (6 μ l H0.6 per assay). *D*, The 164-bp (–102 to +62) *AluI* conalbumin fragment was digested with *Bst*NI, *Mbo*II or *Mnl*I to produce an overlapping set of smaller fragments which were purified directly or subcloned, excised and purified (see text). The numbers in parentheses above line *a* correspond to the location (bp) of restriction endonuclease cleavage sites relative to the cap site (+1). The numbers above the fragments depicted in lines *b-k* correspond to the fragment size (bp). In lines *i, j* and *k* the bracketed numbers correspond to the location of the *Eco*RI and *Hind*III sites on the pBR322 map²⁷ and the dotted lines correspond to pBR322 sequences (see text). The results are expressed as in Fig. 3*B*.

The TATA box region is involved in the 'stable' binding of transcription factor

To locate the conalbumin sequence responsible for stable binding of the transcription factor, various fragments smaller than the 164-bp conalbumin *AluI* fragments were generated by cleavage with *Bst*NI, *Mbo*II and *Mnl*I restriction endonucleases. These fragments were isolated directly by gel electrophoresis (Fig. 4*D*, *b-h*) or by sucrose gradient fractionation, after subcloning into the DNA polymerase I-repaired *Cla*I site of pBR322 followed by excision with *Eco*RI and *Hind*III endonucleases (Fig. 4*D*, *i-k*). The termini of all of these fragments were made blunt by *S*₁ nuclease treatment before being tested in the prebinding assay (Fig. 4, *A-C*). The 106-bp (–44 to +62) and 48-bp (–44 to +4) fragments (Fig. 4*D*, *c, e*, and Fig. 4*A, B*) were at least as efficient as the 164-bp *AluI* fragment in reducing accurate transcription from the Ad2 *Taq*I template. In contrast, the 36-bp (–44 to –8), 70-bp (–8 to +62) and 28-bp (–24 to +4) fragments (Fig. 4*D*, *f-h* and Fig. 4*A, C*) were not significantly more efficient at reducing transcription than were the more distal 58-bp (–102 to –44) and 58-bp (+4 to +62) fragments (Fig. 4*D*, *b, d*, and Fig. 4*A, B*). Comparison of Figs 3*B*, 4*A* and 5*A* shows that the efficiency of a given fragment (here the 164-bp *AluI* fragment) in reducing accurate Ad2 transcription varied somewhat between experiments. However, in all cases, differences between fragments exhibiting specific or nonspecific binding were large enough to distinguish them unequivocally (for all fragments the experiments were repeated several times with different DNA preparations).

The above data indicate that, while being contained within the –44 to +4 sequence, the putative binding region must extend further upstream than position –24 and further downstream than –8. However, these data do not indicate, within these boundaries, which sequences are specifically involved in the formation of the stable complexes.

To assess whether the TATA box sequence could be involved in factor-specific binding, DNA fragments containing *in vitro* generated deletions, known markedly to affect *in vitro* accurate initiation of transcription^{10,16}, were tested in the prebinding assay. Four mutated fragments were used as prebinding DNAs: the conalbumin 160-bp *AluI* fragment with a 4-bp deletion within the TATA box (sequence 5'-TAA-3', instead of 5'-TATAAAA-3'; see ref. 16); the Ad2 major late promoter containing fragments extending to position +197 and in which the sequences upstream from position –32 (Ad –32), –29 (Ad –29) and –21 (Ad –21) have been deleted and replaced by further upstream Ad2 sequences (see Fig. 5 legend and ref. 10; the –32 deletion possesses the intact TATA box 5'-TATAAAA-3', whereas the –29 and –21 deletions have the sequence 5'-TAAAA-3' and a complete deletion of the TATA box, respectively). Note first that the Ad2 major late promoter fragment which contains an intact TATA box seems to be as efficient as the conalbumin *AluI* fragment at forming stable complexes (Fig. 5*B*). Deleting 4 bp within the conalbumin TATA box (Fig. 5*A*) or the first two upstream base pairs from the Ad2 major late TATA box (Fig. 5*B*) reduced the efficiency of factor binding by about 1.6- and 3-fold, respectively, whereas deletion of the entire Ad2 TATA box (to position –21) eliminated the formation of stable complexes (Fig. 5*B*). From these data we conclude that the TATA box sequence has an important role in binding transcription factor to form a stable complex. However, the sequence requirements, in terms of tolerance to deviation from the consensus TATA box sequence^{6,10}, may differ from those previously demonstrated for the production of specific 'run-off' transcripts *in vitro*. The conalbumin 4-bp deletion¹⁶ and the Ad2 major late promoter –29 deletion¹⁰ are indeed known to reduce the corresponding *in vitro* accurate transcription by at least 50-fold. This less stringent requirement for an intact TATA box probably accounts for the finding that, whereas the isolated conalbumin 28-bp (–24 to +4) fragment

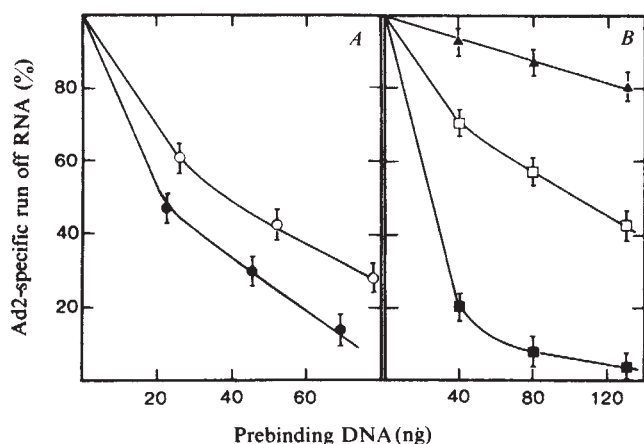


Fig. 5 Deletions of the 'TATA' box sequence affect the formation of preinitiation complexes. Blunt-edged DNA fragments containing deletions in the TATA box sequence of the conalbumin and Ad2 major late promoter regions were tested in the prebinding assay as described in Fig. 2 legend (6 μ l H0.6 per assay). **A**, ●—, The wild-type (TATAAAA), 164-bp (–102 to +62) *AluI* conalbumin fragment; ○—, the mutant (TAA) 160-bp *AluI* fragment containing a 4-bp deletion in the TATAAAA sequence¹⁶. **B**, 470-bp *HindIII* Ad2 fragments containing upstream deletions extending to positions –32 (Ad-32), ■—; –29 (Ad-29), □—; –21 (Ad-21), ▲—; –1 (Ad-1), △—. The Ad2 fragments were excised from the corresponding pMLA derivatives (Fig. 1 of ref. 10) by *HindIII*, purified and *S*₁ nuclease treated before testing. The second DNA added to the assay was, in each case, the Ad2 *TaqI* fragment (130 ng). The results are expressed as in Fig. 3B.

cannot form stable complexes (Fig. 4D, lane *h* and Fig. 4C, see above), the same fragment subcloned in either orientation into the *ClaI* site of pBR322 can function as an efficient prebinding DNA when excised with *EcoRI* and *HindIII* endonucleases (Fig. 4D, lanes *j*, *k*, and Fig. 4C). In these fragments, the 28-bp sequence is preceded either by the A + T-rich pBR322 sequence 5'-TTATCAT-3' (Fig. 4D, lane *j*) or 5'-TATC-3' (Fig. 4D, lane *k*) which occur in the same spatial relationship relative to the cap site as the conalbumin 'wild-type' 5'-TATAAAA-3' sequence (Fig. 4D, lane *a*). For stable binding of transcription factor, these pBR322 sequences probably substitute for the normal TATA box sequence. We also note that a fragment which contains the 80-bp (–24 to +56) conalbumin sequence (cloned into the repaired *ClaI* site of pBR322) and also carries the pBR322 sequences 5'-TTATCAT-3' in place of the normal TATA box sequence, is not more efficient than the analogous 28-bp (–24 to +4)-containing fragment (Fig. 4B, D, *i*, *j*). This result is consistent with the finding that the 48-bp (–44 to +4) and the 106-bp (–44 to +62) fragments demonstrated approximately equal inhibition efficiencies (Fig. 4A, B, Dc, *e*).

Formation of stable preinitiation complexes with partially purified factors in the absence of RNA polymerase B

The results shown in Fig. 2 suggest that RNA polymerase B is not essential for the formation of stable complexes. To address this question more directly, the factors were separated from RNA polymerase in the H0.6 fraction by phosphocellulose chromatography¹¹ and stepwise elution by 0.35 M KCl (P0.35), 0.5 M KCl (P0.5) and 1.0 M KCl (P1.0). As determined by ³H-amanitin binding (data not shown) P0.1 (the 0.1 M KCl flow-through fraction) contained 3% of the RNA polymerase, P0.35 contained 95% and the other 2% was in P0.5. For specific transcription from conalbumin templates (not shown) or Ad2 major late template (Fig. 6A, lanes *a*, *b*) the P0.5 and P1.0 fractions were usually (but see below) not sufficient; also needed was the H0.1 heparin-Ultrogel fraction (not shown) or the DE0.35 fraction obtained by elution with 0.35 M KCl of the H0.1 fraction absorbed to a DEAE-cellulose column equili-

brated as described in Fig. 1 legend. Note that the DE0.35 fraction was inhibitory when used in excess of 3 μ l per assay (Fig. 6A, lanes *b*, *e*–*g*) and was less efficient when added after the initial preincubation period (compare lanes *b*–*d* of Fig. 6A). Because this latter decrease in activity was independent of the length of the second preincubation period (not shown), it probably relates, at least in part, to instability of the phosphocellulose fractions, while preincubating in the absence of the DE0.35 fraction (compare also lanes *b*, *c* of Fig. 6B, C).

Using fractions DE0.35, P0.5 and P1.0, specific transcription was totally dependent on addition of calf thymus RNA polymerase B after the preincubation period (Fig. 6B, C, lanes *a*, *b*). Preincubation of the combined P0.5, P1.0 and DE0.35 fractions or the combined P1.0 and DE0.35 fractions with an Ad2 major late template containing the TATA box (Ad-32, 197 nucleotide run-off RNA) essentially eliminated specific transcription from a second template (con, 230 nucleotide run-off RNA) (Fig. 6B, lanes *d*, *f*). Prebinding with the DE0.35 fraction alone or in combination with the P0.5 fraction (Fig. 6B, lanes *g*, *e*) resulted in a ratio of specific adenovirus and conalbumin run-off RNAs identical to that seen with simultaneous template addition (Fig. 6B, lane *c*). That it is binding at the Ad2 major late TATA-box sequence which, after preincubation with the P1.0 and DE0.35 fractions, precluded transcription of the conalbumin template was indicated by the presence of the conalbumin-specific transcript when the –32 deletion-containing Ad2 template was replaced by the template lacking the corresponding TATA box (Ad2 –21, see legends to Figs 5, 6) (Fig. 6B, compare lanes *e*–*g* with lanes *j*–*l*). These results show that stable preinitiation complex formation at the Ad2 major late promoter TATA-box region does not require RNA polymerase B or the P0.5 fraction.

To determine if both the DE0.35 and P1.0 fractions were required for stable complex formation, we performed an experiment (Fig. 6C) identical to that shown in Fig. 6B except that the DE0.35 fraction was added during the second preincubation period simultaneously with the conalbumin template. No stable complex formation was observed with the P1.0 fraction when the DE0.35 fraction was not present during the initial preincubation period: the ratio of specific run-off RNAs seen in lanes *d* and *f* is identical to that seen with simultaneous template addition (lane *c*) (ratios rather than absolute amounts of specific RNA should be compared because phosphocellulose factors are clearly unstable when incubated in the absence of DNA and/or DE0.35 fractions, see above). We conclude that factors contained in the DE0.35 and P1.0 fractions are required for stable preinitiation complex formation. An additional factor(s), present in the P0.5 fraction, is then required for productive initiation of transcription by RNA polymerase B.

Although the DE0.35 factor activity did not bind to heparin-Ultrogel or phosphocellulose in the presence of 0.1 M KCl (not shown), efficient specific transcription and stable complex formation were nonetheless observed in assays containing only the H0.6 fraction (Figs 2–5). Furthermore, we have occasionally observed a low level of specific transcription using the P0.5 and P1.0 fractions only and, in some of these instances, stable preinitiation complex formation was observed using the P1.0 fraction alone¹⁷. Thus, it appears that a small proportion of the factor(s) contained in H0.1 could in some instances co-chromatograph with the P1.0 factor(s). In our hands, two heparin rechromatography steps were required to render the H0.6 fraction completely dependent on addition of H0.1 for specific transcription. Given the results of Fig. 6C, these observations indicate that the factor(s) present in the DE0.35 fraction may function through a direct interaction with factor(s) contained in the P1.0 fraction. Finally, we note that the P1.0 fraction was clearly limiting with respect to preinitiation complex formation, as further addition of the P1.0 fraction, but not DE0.35, P0.5 or RNA polymerase B fractions, concomitant with the conalbumin template, restored efficient specific transcription of this template (E.R.M. and B.L.D., unpublished results).

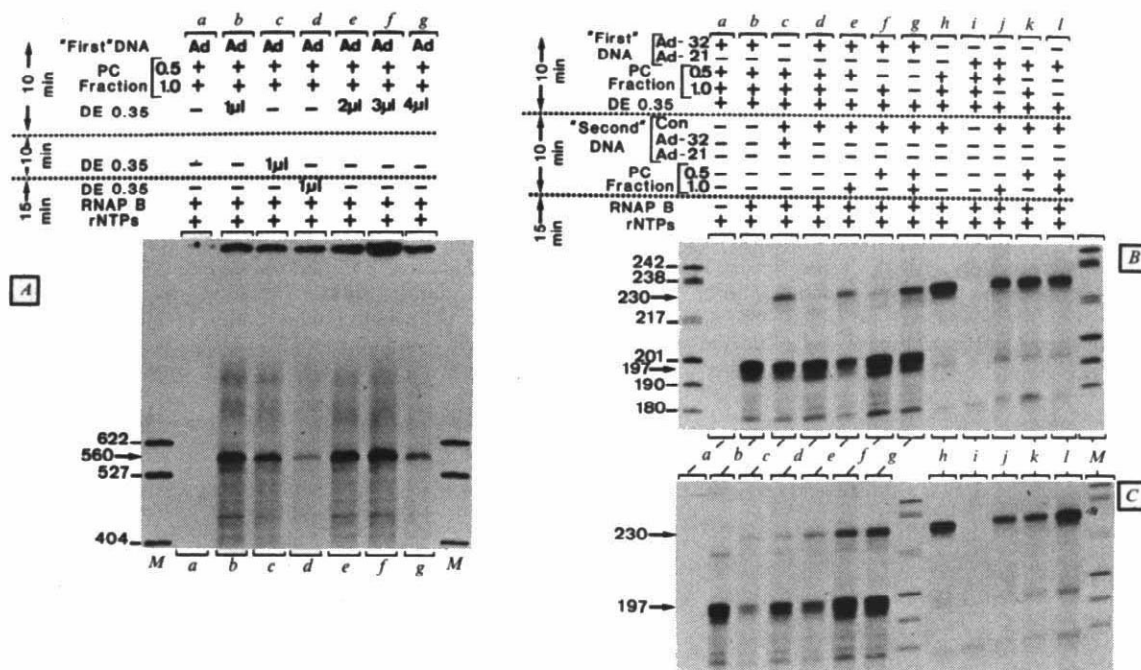


Fig. 6 Formation of stable complexes with partially purified factors in the absence of RNA polymerase B. The P0.5 and P1.0 fractions were obtained by chromatography of the H0.6 fraction (4 ml, see Fig. 1) on a column (5 ml) of phosphocellulose essentially as described by Matsui *et al.*¹¹ for chromatography of an S100 extract. The DE0.35 fraction was prepared from the H0.1 fraction as described in the text. **A**, Various combinations of the DE0.35 (0–4 μl), P0.5 (5 μl) and P1.0 (5 μl) fractions were tested for specific transcription activity with the wild-type Ad2 major late template (Ad, 150 ng of the *TaqI* fragment, 560 nucleotide run-off RNA, see Fig. 1 legend) according to the preincubation scheme shown at the top of the figure (the lengths of the incubation periods are indicated in min). In these assays (30 μl final volume), the initial preincubation volume was 21 μl (see legends to Figs 1, 2) and no second DNA template was used. **B**, Various combinations of the DE0.35 (1 μl), P0.5 (5 μl) and P1.0 (5 μl) fractions were preincubated for 10 min in 21-μl reaction mixtures (see legends to Figs 1, 2) with 200 ng of either the Ad2-32 (lanes a, b and d–g, 197-nucleotide run-off RNA, see Fig. 5 legend) or the corresponding TATA box-deleted Ad2-21 template (lanes i–l, no specific run-off RNA, see Fig. 5 legend) as indicated at the top of the figure. No first DNA template was used in lanes c and h. During the second preincubation period (10 min), conalbumin (con, 150 ng of the 354-bp *MspI* fragment cloned into the *Clal* site of pBR322 and excised with *EcoRI* and *HindIII* endonucleases and having a 230-nucleotide run-off RNA, refs 13, 17) or Ad2-32 (200 ng) DNAs were then added to the assays as second templates along with, in some cases, the P0.5 (5 μl) or P1.0 (5 μl) fractions. **C**, Identical to **B**, except that the DE0.35 fraction (1 μl) was added during the second preincubation period. In **A–C**, transcription was initiated by the addition of rNTPs and 0.01 units of calf thymus RNA polymerase B (30 μl final reaction volume, see legends to Figs 1, 2), except in lane a of **B** and **C** where no RNA polymerase B was added. All DNA fragments were made blunt-ended by repairing with DNA polymerase I (Klenow fragment) followed by extraction with phenol/ CHCl_3 and sucrose gradient centrifugation. The results presented in **A–C** were obtained using the same preparation of factor-containing fractions and each panel corresponds to a single gel (12-h exposures, with intensifying screens). The results shown here have been reproduced in six independent experiments using four different factor preparations. *M*, size markers.

Conclusion

Our results demonstrate that transcription factor(s) specific for RNA polymerase B can form specific preinitiation complexes with DNA fragments containing class B promoter sequences. These complexes are strikingly more stable than those formed with non-promoter sequences and are, therefore, readily distinguishable. RNA polymerase B is not required for specific complex formation and, once bound at a 'promoter site', the transcription factor(s) is functionally committed to specific initiation from this promoter for at least 20 min in our assay conditions (minus NTPs).

The -44 to +4 conalbumin promoter region contains the essential sequence elements for the formation of specific stable complexes. The results obtained with DNA fragments lacking the TATA box region or with deletions within this sequence indicate clearly that it is involved in the formation of stable complexes with both Ad2 major late and conalbumin promoter regions. It seems, however, that the requirement of an intact TATA box for stable binding of factor(s) is less pronounced than that for accurate and efficient initiation of run-off transcription. The pBR322 sequences TTATCAT or even TATC can replace, at least in part, the genuine conalbumin TATA box for the formation of stable complexes, whereas we found that they cannot direct accurate transcription *in vitro* (B.L.D., unpublished results). These results suggest that the TATA box

might have several roles in the initiation process, possibly through interactions with several components of the transcription machinery, and pose the question as to whether the same factor(s) could form stable preinitiation complexes with promoter sequences which lack a 'canonical' TATA box, such as those of the Ad2 EIIa early promoter (for refs, see refs 18, 19).

The result obtained with the isolated, -44 to -8, 36-bp conalbumin fragment (Fig. 4A) suggests that besides the TATA box sequence, additional sequences, closer to the cap site, could be involved in the formation of stable complexes. However, we have found (B.L.D., unpublished result) that a 610-bp fragment containing a direct repeat of the Ad2 major late -32 to -12 sequence inserted in a pBR322 fragment (*HindIII*-*SphI* fragment, see ref. 14) is efficient at forming stable complexes, which indicates that the requirement for sequences downstream from position -8 may not correspond to a sequence-specific interaction. It has been shown that sequences located upstream from the TATA box can be important for initiation of transcription *in vivo* and even *in vitro* (see for example, refs 6, 7, 20–22). Such sequences are not absolutely required for the formation of stable complexes, at least in our present assay conditions, but their possible involvement is suggested by the results shown in Fig. 5A, B, where a deletion within the TATA box has a more pronounced effect in the case of the Ad2 fragment, which lacks the wild-type sequences upstream from -32, than for the

conalbumin fragment which possesses the wild-type sequences up to -102.

At least two factors contained in the P1.0 and DE0.35 fractions are required for the TATA box-dependent formation of stable preinitiation complexes. As the P1.0 factor alone, but not the DE0.35 factor (B.L.D., unpublished results) binds to DNA in the presence of 0.05 M KCl, it seems that the latter could favour the formation of stable preinitiation complexes by interaction with the P1.0 factor. Further studies will show whether these factors promote accurate initiation of transcription directly by interaction with the transcription machinery, or indirectly by modifying the DNA structure, thereby providing a specific initiation signal. We note that such an initiation factor(s) which forms stable complexes with specific promoter

DNA sequences and does not co-purify with RNA polymerase, has very different properties from the classical prokaryotic sigma initiation factors^{23,24}.

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LETTERS TO NATURE

VLA source counts at 6-cm wavelength

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Radio sources can have such high luminosities that they are detectable at great distances. They may therefore be important tracers of the structure and evolution of the early Universe. They apparently evolve in time; compared with the present epoch, strong radio sources are orders of magnitude more common at redshifts z greater than unity^{1,2}. Direct measurements of the number of radio sources observed as a function of their flux places constraints on both their evolutionary behaviour and on the cosmological models. To extend the present source counts to lower flux limits, the Very Large Array (VLA) of the National Radio Astronomy Observatory was employed. Thirteen widely separated fields at high galactic latitude were mapped at the VLA at 6 cm. The fields are 12.8 arc min square, and the 5 σ noise levels extend below 1 mJy. The resulting differential source counts were $\Delta N/\Delta N_0$ ($0.5 \text{ mJy} < S < 3.0 \text{ mJy}$) = 0.0384 and $\Delta N/\Delta N_0$ ($3.0 \text{ mJy} < S < 10.0 \text{ mJy}$) = 0.178. These results confirm the deviation at low flux levels from the uniform $N = 60 S^{-1.5} \Omega$ distribution.

Radio source count data in combination with a radio luminosity function place constraints on cosmological models^{1,2}. The earliest radio source counts were inconsistent with any model of the Universe in which the co-moving density of radio sources was fixed³, and it is now widely accepted that radio sources do evolve. Longair suggested, as early as 1966, that the evolution

was limited to powerful sources⁴. Subsequent observations have refined the radio luminosity function and it now appears that steep-spectrum sources evolve more strongly than non-steep spectrum sources^{3,5,6}. Steep-spectrum sources which are unresolved at arc second scales seem to evolve more like extended steep-spectrum sources than like compact sources^{3,7,8}.

Several papers have been published recently based on a sample of 168 radio sources observed at 2.7 GHz covering 4.05 sr and complete to 1.5 Jy (refs 5, 7). With this sample it is possible to eliminate some models, but more observations are required; in particular, "...there remains the need to improve the definition of the counts... at mJy levels for the higher frequencies."⁹ The lowest flux level direct 5-GHz source count data published to date extend to 4.5 mJy (refs 10, 11). The present study improves on these measurements, providing 5 GHz source count data, obtained at the VLA, extending below 1 mJy.

Thirteen fields were mapped at the VLA at 6-cm wavelength in September 1981. These fields are at high galactic latitude ($|b| > 20^\circ$) to avoid sources in our Galaxy, and at widely separated points on the sky to avoid sampling in a single cluster or supercluster. The fields were observed at antenna elevation angles $> 40^\circ$ to avoid cross-talk and partial shadowing in the closely spaced D-array of the VLA. The fields were centred on even hour angles and therefore are effectively a random sample.

The 13 VLA maps differ in their flux limits. Furthermore, the single antenna beam (primary beam) pattern falls off significantly within a single D-configuration map, and thus the minimum detectable flux in a given map varies with position. To allow for these flux limit differences, each map was divided into 11 rings, such that the flux limit within a given ring is sufficiently constant and well determined. The corners of the maps (256×256 , 3 arc s cells) are below the level where the primary beam pattern correction (ref. 12 and P. Napier, personal communication) was applied to the remaining rings.

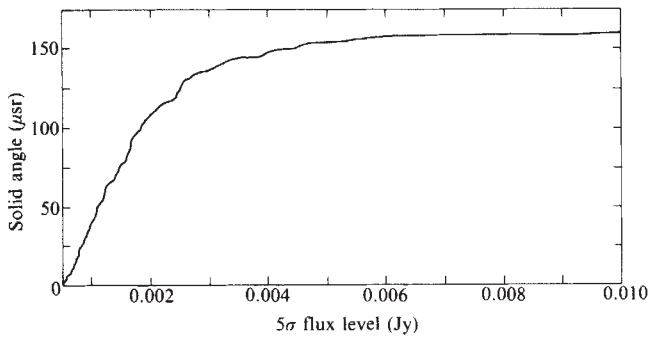


Fig. 1 The observed angle $\Omega(S > 5\sigma)$, in μsr , as a function of the 5σ detection flux limit.

The r.m.s. flux in each ring was computed, and peaks of 5σ or greater were taken to be sources. This method may systematically miss extended sources, as it is the peak flux and not the integrated flux that determines the detection of a source, but in view of the 3 arc s cell size and the 6-cm observing wavelength, this is probably not a serious problem. The sources identified by the formal 5σ criterion were exactly the same as those chosen by simply examining the contour maps. Figure 1 shows the solid angle $\Omega(S > 5\sigma)$ as a function of the 5σ detection flux limit for all of the 143 rings up to the 10 mJy level.

The observed fields and detected sources are listed in Table 1. None of these sources had optical counterparts on the Palomar Sky Survey prints.

The field 1500+400 contains four 'objects' labelled *a*, *b*, *c* and *d*. *a*, *b*, and *c* are co-linear, and their separation is consistent with the angular sizes of the double-lobe radio galaxies. It is assumed that these three objects are in fact one radio galaxy, and that the fourth, *d*, over 2 arc min away with no apparent connecting bridge, is an independent source. Future observa-

Table 1 The observed fields with coordinates, fluxes, signal-to-noise ratio and ring number for each source in the $0.5 \text{ mJy} \leq S \leq 10.0$

Field	RA	Dec	Flux (mJy)	S/N	Ring no.
0700+500	06h 59min 50.690s	50° 02' 10.73"	2.84	18.3	4
	06 59 57.592	49 56 43.41	1.50	8.8	5
0800+400	07 59 44.160	39 59 33.87	0.82	6.2	4
	08 00 11.456	40 02 05.95	4.29	33.7	5
	08 00 22.879	39 59 27.24	5.52	30.9	7
0900+400					
0900+800					
1000+400					
1000+800					
	09 58 53.030	79 57 17.95	3.61	10.9	6
	10 01 10.088	79 59 27.21	4.43	15.4	5
1100+400					
1100+785					
1100+800	11 02 01.637	78 32 12.43	2.71	5.5	10
	10 59 40.671	79 59 39.12	0.95	5.6	1
	10 59 43.773	80 05 51.63	4.23	7.8	9
1200+800					
1300+400					
1300+800	13 00 14.709	40 01 53.05	2.38	9.5	5
	13 00 06.240	80 06 19.02	3.79	7.4	10
1500+400					
(d) 14 59	54.969	39 56 13.17	6.01	21.7	6
(a) 15 00	05.793	39 57 55.26	4.78	30.7	3
(b) 15 00	08.436	39 57 38.88	4.65	23.2	4
(c) 15 00	11.302	39 57 04.01	3.24	13.6	5

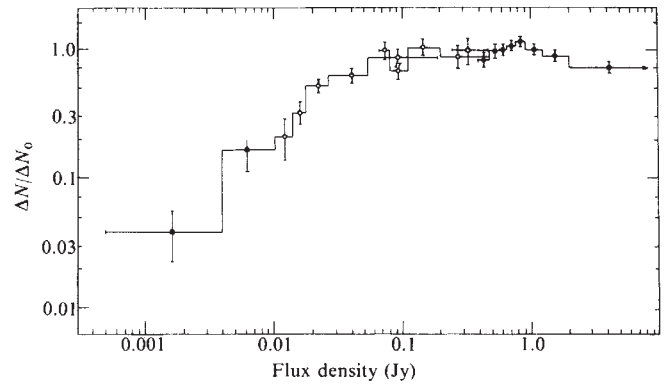


Fig. 2 The differential source counts compiled by Pauliny-Toth¹⁰. The two shaded boxes at the lower left are the new data presented here. The counts have been normalized to the integral count $n_0 = 60 \text{ S}^{-1.5}$.

tions of this source with a longer integration time will check this assumption, but our conclusions are insensitive to the outcome. This three-component source is treated as having a total flux equal to the integrated flux of its three components, which is 12.67 mJy. There is an insufficient number of sources with $S > 10 \text{ mJy}$ to provide good statistics, and these sources are not included in the source counts or Table 1.

If $n_0(S)$ is defined as the number of sources per sr with flux greater than S , then $(dn_0/dS) dS$ is the differential number of sources per sr between flux S and $S + dS$. The total number of sources between fluxes S_1 and S_2 is then

$$\Delta N_0(S_1 \leq S \leq S_2) = \int_{S_1}^{S_2} \Omega(S) \frac{dn_0}{dS} dS \quad (1)$$

where $\Omega(S)$ is the solid angle observed to a lower flux limit S . The standard 6-cm uniform Euclidean source distribution is chosen to be $n_0(\text{sr}^{-1}) = 60S_{\text{Jy}}^{-1.5}$. Thus

$$\Delta N_0(S_1 \leq S \leq S_2) = 90 \int_{S_1}^{S_2} \Omega(S) S^{-2.5} dS \quad (2)$$

where $\Omega(S)$ is in sr and S is in Jy. This standard notation uses '0' subscripts to denote theoretical values which will be used to normalize the measurements. Equation (2) was computed with the $\Omega(S)$ from Fig. 1 for the flux ranges 0.5–3.0 mJy, and 3.0–10.0 mJy. The observed number of sources per flux bin is $\Delta N(0.5 \text{ mJy} \leq S \leq 3.0 \text{ mJy}) = 6$ and $\Delta N(3.0 \text{ mJy} \leq S \leq 10.0 \text{ mJy}) = 7$ resulting in the normalized source counts

$$\frac{\Delta N}{\Delta N_0}(0.5 \text{ mJy} \leq S \leq 3.0 \text{ mJy}) = 0.0384 \pm 0.016 \quad (3a)$$

$$\frac{\Delta N}{\Delta N_0}(3.0 \text{ mJy} \leq S \leq 10.0 \text{ mJy}) = 0.178 \pm 0.067 \quad (3b)$$

where the uncertainties are Poisson sampling errors. These results are insensitive to individual source flux errors. The maximum-likelihood estimations^{13,14} call for negligible corrections to these data, and have not been applied.

The new 5-GHz source count results presented here are shown in Fig. 2 along with previous results reported and collected by Pauliny-Toth *et al.*¹⁰. The new results confirm the continued deviation at low flux levels from the uniform $N(S) = 60 S^{-1.5}$ Ω distribution.

The source counts shown in Fig. 2 follow a reasonable extrapolation of the trends shown by Pauliny-Toth *et al.* Indirect source count measurements, based on $P(D)$ statistical analyses^{15–17}, are also roughly consistent with these new direct counts. There is no evidence for a dominant population of intrinsically weaker but common radio sources lurking below the main population; the general decline in source counts is a cosmological fact that must be reckoned with. A definitive study requires knowledge of the luminosity function; towards that end we are

making high resolution studies of these sources with the VLA and pursuing a programme of optical identification.

High resolution observations are now underway at the VLA to determine source structure at 5 GHz, and spectral index using the 20-cm system. Optical observations will also be required¹⁸. These data will be checked for consistency against Laing and Peacock's P - α relation for steep-spectrum sources¹⁹.

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Three unusual cataclysmic variable stars

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A recent survey for variable objects¹ using an automated search procedure detected a number of variables for which it was only possible to give a preliminary classification. Many of the sample have now been observed spectroscopically on a large telescope and definite classifications made. Noticeable among these are three cataclysmic variables which according to well defined criteria form a complete sample of such objects. All these cataclysmic variables are very faint, and two of them do not readily fit into any existing category.

The procedure for detecting the variables, described in detail elsewhere^{1,2}, involved measuring a sequence of Schmidt plates with the COSMOS measuring machine. The resulting data set consisted of B magnitudes for about 10^5 images from a set of eight plates. In the first instance a rather simple algorithm was used to locate a sample of the most variable objects, and preliminary classifications were made on the basis of the type of variability and appearance on an objective prism plate. Four classes were identified comprising RR Lyrae stars, candidate BL Lac type objects, quasars and objects with a flat blue continuum and a sharp cutoff in the blue.

To check the initial classifications several sample members¹ were observed spectroscopically with the IPCS on the Anglo-Australian telescope (AAT) in August 1981. The spectra were taken through gaps at the end of a cloudy night and it was unfortunately not possible to obtain a flux calibration. Four sample members V1, V4, V5 and V6, stand out in amplitude

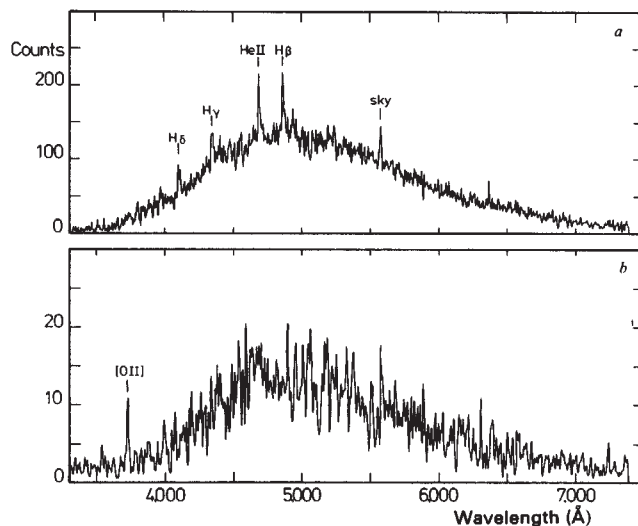


Fig. 1 AAT spectra of: a, V1; b, V5. Resolution is $2.0 \text{ \AA}$ per channel; instrumental response has not been removed.

from the remainder of the sample, with $\delta B > 1.35$, the next largest being V3 with $\delta B = 1.14$. Among the four large amplitude variables, V4 turned out to be an A star and is now classified as an RR Lyrae variable. V1 with a B amplitude of 2.85 mag in a few days and an apparently featureless blue continuum was originally classified as a possible BL Lac candidate. The AAT spectrum shown in Fig. 1a was taken at maximum and shows the Balmer series in emission and strong He II $\lambda 4,686$; the systemic velocity is $< 100 \text{ km s}^{-1}$. The initial classification for V5 was rather uncertain, the only significant feature of the objective prism spectrum being a sharp cutoff in the blue. The apparently stellar image varied by 1.5 mag in a few days. The AAT spectrum in Fig. 1b is unfortunately not of very good quality due to the faintness ($B \sim 20$) of the object when it was observed, although one feature is outstanding, a very strong [O II] $\lambda 3,727$ line, which is probably responsible for the apparent cutoff seen on the objective prism plate. Significantly, no Balmer series emission lines are visible, and the noise in the spectrum puts a conservative upper limit on their presence at about one-third the strength of the $\lambda 3,727$ line. The systemic velocity is $< 100 \text{ km s}^{-1}$. V6, the final member of the large amplitude group, was observed on the AAT by T. Shanks in 1982 while the object was at maximum. The spectrum is typical of that of a dwarf nova in outburst with broad H absorption partly filled in with emission and broad He I $\lambda 4,471$ absorption.

Light curves were obtained from a sequence of 15 IIIaJ plates, taken with the UK 1.2-m Schmidt between 1975 and 1981, and including the plates on which the objects were first discovered. The photometric accuracy of the measures is about 0.09 mag. The observations cover time scales from years to days, and are shown for V1 and V5 in Fig. 2. In order to pin down the day to day variations of the objects, both were observed for 10 days in October 1981, with the McMullan electronographic camera on the Danish 1.5-m telescope at La Silla, Chile. A 10-min exposure in B was taken at the start of each night. In this case the photometric accuracy was about 0.05 mag, the light curves being shown in Fig. 3. Additional photometric material was obtained from a sequence of 25 UKST IIIaF plates taken almost every night in two runs of two weeks, the runs being separated by about two weeks, in 1980. They show V1 at $R \approx 16.9$ on all plates and V5 fluctuating by about 0.5 mag around $R = 18.5$. V6 underwent an outburst during this period and the light curve is shown in Fig. 4. $B - R$ colours for the objects from plates taken on the same night were 0.92 for V1, 0.75 for V5, and 1.69 for V6, V1 being at maximum and the other two near minimum. Finding charts for

Table 1 Photometric and spectroscopic data of the three cataclysmic variables

Object	RA (1950)	Dec (1950)	<i>B</i> (at max)	Amplitude	Spectrum
V1	21 h 34 m 45.2 s	-43° 55' 46"	17.87	2.85	Strong H and He II emitted
V5	21 25 23.2	-42 45 37	18.30	1.50	Strong [O II] emitted
V6	21 25 56.0	-42 42 10	16.47	2.06	Broad He and He I absorbed, H emitted

these three objects have already been published¹, and the photometric and spectroscopic data are summarized in Table 1.

The classification of V1 and V5 has turned out to be surprisingly difficult. The spectrum of V1 in Fig. 1a closely resembles that of an old nova such as CP Pup, with very strong He II and the Balmer series in emission although H α is very weak and presumably implies a strong self reversal. The photometric variations, however, are unusual for this type of system. Altogether, some 50 observations are now available for the object and all show it in one of two states, corresponding to $B \approx 18$ and $B \approx 21$. All observations up to the final one in 1977 show the object at minimum, including the Palomar sky survey, ESO B survey, and a poor quality early SRC plate. During 1978 the magnitude fluctuated between the two extremes on a time scale of <3 days, and since then has remained more or less steadily at maximum. Until the long series of R plates it seemed possible that a period could be fitted to the observations, although the fact that it spent about a third of its time at minimum made this difficult. It now seems that periodic variations consistent with all the data can probably be ruled out. Taken at face value, this type of behaviour is most unusual for such an object. The increase in brightness by 3 mag is not of a similar nature to normal cataclysmic variable outbursts when the emission lines tend to be washed out by the continuum. This appears to be a definite shift from a faint to a bright state,

with an intermediate period of fluctuation. At the moment the indications are that once established the respective states last for years.

There is clearly some qualitative similarity between V1 and some X-ray sources which appear to have bright and faint states. Z Cam stars also exhibit similar behaviour, but the strength of the $\lambda 4,686$ line in the spectrum of V1 is unusual for a dwarf

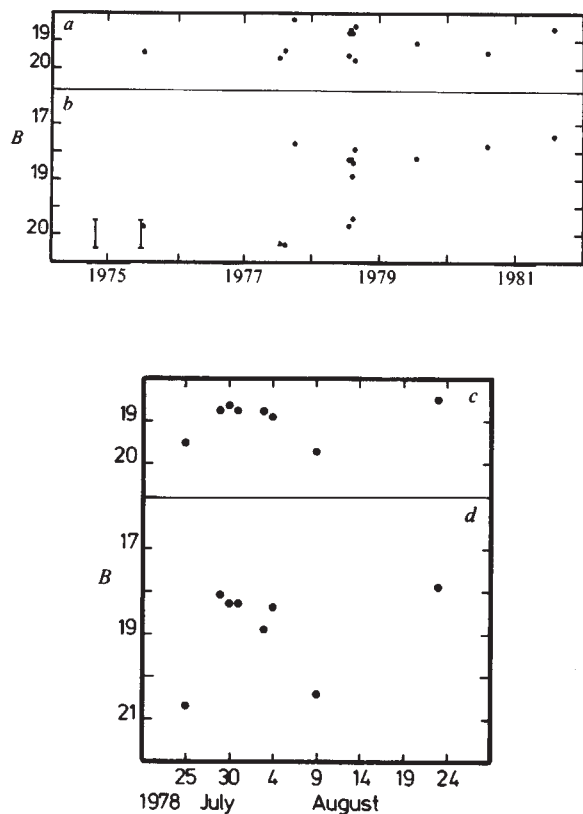


Fig. 2 Light curves for V5 (a, c) and V1 (b, d) between 1975 and 1981 (a, b) from UKST IIIA plates and, enlarged scale for 1978 (c, d).

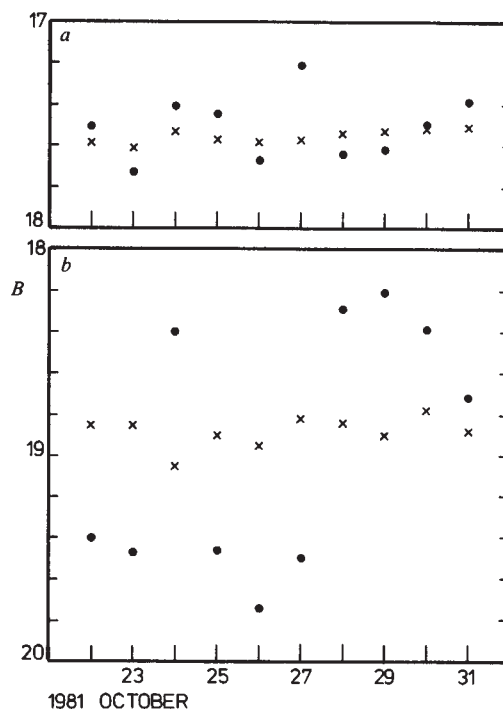


Fig. 3 Light curves for V1 (a) and V5 (b) from 8-cm McMullan camera on Danish 1.5 m telescope. Crosses show photometry of comparison stars.

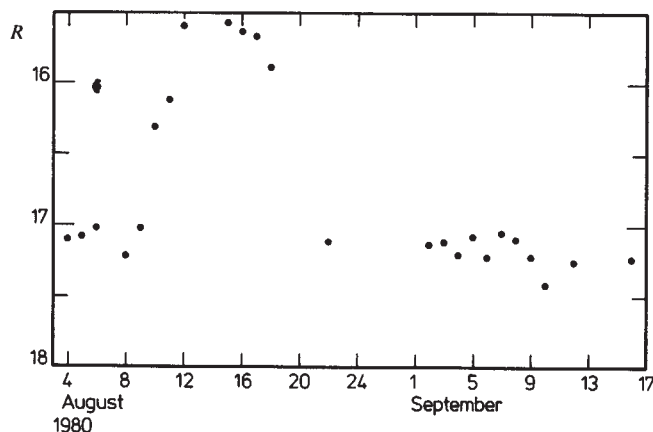


Fig. 4 Light curve for V6 for August and September 1980 from UKST IIIA plates.

nova. Among the X-ray sources, V1 is perhaps closest to AM Her type objects, although the duration of the states of say VV Pup appears to be very different³. The object is clearly a good candidate for an X-ray emitter, although it does not appear in any of the existing catalogues. This could well be because it is fainter and presumably further than known objects of a similar type.

V5 appears to show a contradictory set of observational features, comprising a faint starlike appearance, varying by a magnitude in a few days, but with a zero-redshifted $\lambda 3,727$ line and no detectable hydrogen lines. The most likely classification would appear to be a DQ Her type system at a sufficient distance for the nebula lines to be superimposed on the stellar continuum⁴. The variation is presumably associated with the central star and is to be expected in an old nova. The strength of the $\lambda 3,727$ line compared with $H\beta$ is unusual but not unprecedented in such systems. If the continuum is from a late type star then $H\beta$ from a nova shell could well be swamped.

The rather arbitrary selection criteria and very small numbers in this sample of cataclysmic variables must inevitably limit its use for statistical purposes. It is nonetheless the first of its type complete to objectively set limits and can provide a first step in assessing the distribution and frequency of occurrence of these objects. The sample may be considered complete in the following sense. It contains in principle all cataclysmic variables in an area of 16 deg^2 with $B_{\text{max}} < 21$, and which varied by more than 1.5 mag in B on the set of eight plates covering the 2 years. These criteria are perhaps not as arbitrary as they appear, because follow up searches on about 40 additional plates have failed to reveal any new cataclysmic variables, suggesting that the sample is quite well-defined. In fact a considerable number of RR Lyrae stars have now been found, but none of them, and indeed no other variable detected in the field has an amplitude greater than $\delta B = 1.5 \text{ mag}$.

Few estimates of the distribution of cataclysmic variables have been published, and probably the best basis for statistical comparison is the sample of Vogt and Bateson⁵. They find a total of 78 dwarf novae brighter than $m_{\text{pg}} = 13.5$ at maximum, with $\delta < +30^\circ$. This is equivalent to 0.04 in the 16 deg^2 of the present survey. If we follow Vogt and Bateson's estimate⁵ that their stars will be 17–18 mag at minimum we may very roughly conclude on the basis of the one dwarf nova found in the present survey, that the numbers increase by a factor of ~ 25 in 3–4 mag, or a factor of 2–3 per mag. No similar survey for nova-like variables exists, but if we take Warner's⁶ compilation of well studied examples of cataclysmic variables to imply their numbers are roughly equal, then approximately the same rate of increase would apply. Perhaps a more significant aspect of the sample was that the one dwarf nova detected was so bright, some 2.5 mag brighter than the sample limit at minimum. This may indicate a cutoff in numbers associated with the fall off in disk population.

Due to the comparative rarity of cataclysmic variables the compilation of a statistically adequate sample will be a laborious process, involving several Schmidt fields. It is intended to continue the search in the present field for smaller amplitude examples but the likelihood of finding such objects seems small. The objects which were found all have rather moderate amplitudes, and are not typical of the general run of cataclysmic variables.

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Accelerator mass spectrometry measurement of cosmogenic ^{26}Al in terrestrial and extraterrestrial matter

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Cosmogenic ^{26}Al (half life = $7.2 \times 10^5 \text{ yr}$) and ^{10}Be ($1.5 \times 10^6 \text{ yr}$) are produced continually in the atmosphere by cosmic rays. They are fixed on aerosol particles and, after a relatively short time ($\sim 1 \text{ yr}$) precipitate to the Earth's surface, and from there into various geophysical reservoirs. The possibility of using this pair of isotopes as a dating tool, independent of cosmic ray intensity variations, was discussed some time ago¹. However, except for several rare exceptions^{2,3} the very low activities of these isotopes, especially ^{26}Al , have dissuaded workers from such studies by classical radioactive counting techniques. The development of accelerator mass spectrometry offers to change this situation dramatically. Cosmogenically produced ^{10}Be has already been measured by several groups using accelerator mass spectrometry⁴. While the technique has also been used to detect artificially produced^{5–7} and extraterrestrial⁸, ^{26}Al the $^{26}\text{Al}/^{27}\text{Al}$ ratio in each case was several orders of magnitude greater than that expected in natural terrestrial samples. We report here the first accelerator mass spectrometry measurements of ^{26}Al in natural terrestrial samples, and give a value for the atmospheric production ratio of $^{26}\text{Al}/^{10}\text{Be}$, an essential parameter for use of this pair as an absolute dating tool. To demonstrate the potential of our technique for measuring ^{26}Al in small quantities of extraterrestrial material, we have also measured this isotope in milligramme-size samples of lunar soil. Finally, by measuring the absolute $^{26}\text{Al}/^{27}\text{Al}$ ratio of two samples whose radioactive disintegration rate was known, we have obtained an independent check on the ^{26}Al half life.

The first step in testing a potential $^{26}\text{Al}/^{10}\text{Be}$ dating scheme is to determine the atmospheric production ratio. In selecting samples for such a determination, we were guided by two criteria: (1) that they come from as near the site of production as possible, to reduce the possibility of fractionation between the two nuclides; (2) that the quantity of ^{27}Al be minimal, so as to maximize the $^{26}\text{Al}/^{27}\text{Al}$ ratio for measurement on the accelerator. On the basis of these criteria, we chose stratospheric air filters as the most suitable samples. (Although we considered rain samples, here the ^{27}Al concentration in our samples reduced the $^{26}\text{Al}/^{27}\text{Al}$ ratio $< 5 \times 10^{-14}$.)

The stratospheric air filter samples were collected as part of the US Department of Energy project Airstream. Relevant parameters of the sampling conditions are given in Table 1, and further details in ref. 9. The filters were dissolved in the presence of 1-mg quantities of ^9Be and ^{27}Al carrier solutions, and the two elements extracted by solvent extraction, using a procedure similar to that described earlier¹⁰. The Be and Al were then separated on an ion exchange column (Dowex 50 $\times$ 8, 200 mesh), before being converted to oxides.

The accelerator measurements were performed on the University of Pennsylvania's tandem Van de Graaff accelerator, details will be presented elsewhere. However, an essential feature of the procedure was the use of a new caesium sputter ion source, similar to that described in ref. 11, which permits currents of several hundred nano-amperes of Al^+ ions from aluminium oxide. An additional modification to the system

Table 1 ^{26}Al and ^{10}Be in samples studied

Sample (size)	Measured $^{26}\text{Al}/^{27}\text{Al}$ ($\times 10^{-13}$)	^{26}Al atoms in sample ($\times 10^7$)	Measured $^{10}\text{Be}/^9\text{Be}$ ($\times 10^{-13}$)	^{10}Be atoms in sample ($\times 10^7$)	$^{26}\text{Al}/^{10}\text{Be}$ ratio
Air filters					
1242: 15-km altitude 58°–65°N (347 m ³)	6.81 $\pm$ 1.02	1.52 $\pm$ 0.23	521 $\pm$ 37	382 $\pm$ 27	(3.98 $\pm$ 0.67) $\times 10^{-3}$
1310: 19.2-km altitude 62°–71°N (191 m ³)	2.90 $\pm$ 0.87	0.65 $\pm$ 0.19	267 $\pm$ 19	195 $\pm$ 14	(3.33 $\pm$ 1.00) $\times 10^{-3}$
Lunar fines*					
61241: surface (1.84 mg)	33.7 $\pm$ 4.4	16.9 $\pm$ 2.2	3.38 $\pm$ 0.27	2.48 $\pm$ 0.20	6.80 $\pm$ 1.04
61221: 30–35 cm depth (2.14 mg)	26.9 $\pm$ 4.3	13.5 $\pm$ 2.2	5.21 $\pm$ 0.42	3.82 $\pm$ 0.31	3.53 $\pm$ 0.64
Blank					
Reagent BeO or Al ₂ O ₃	≤ 0.08	—	≤ 0.15	—	—

* 30–80 μm size fraction.

described for ^{10}Be measurements¹², was the use of a second analysing magnet. This was found necessary to reduce background due to ^{27}Al scattered from residual gas in the 90° analysing magnet. ^{27}Al transmission efficiencies were typically 12% and negative ion formation efficiencies $\sim 0.05\%$.

All ^{26}Al measurements were made relative to a standard, prepared by adding ^{27}Al to a standardized ^{26}Al solution (obtained from LMRI, Saclay, France) to give a known $^{26}\text{Al}/^{27}\text{Al}$ ratio, and then converting this to Al_2O_3 . Both standard and unknown Al_2O_3 samples were mixed with silver (in a weight ratio of $\sim 1:4$) for measurement on the accelerator.

The ^{10}Be measurements have been made using the procedure described previously¹², and relative to a standard prepared by the technique described in ref. 13.

The results of the accelerator measurements are summarized in Table 1. In general, samples were measured several times. Uncertainties have been estimated either from statistics on the number of events counted, or from reproducibility of these multiple measurements, whichever was larger.

The measurement on filter 13130 was made on a sample in which BeO and Al_2O_3 were not separated, and thus the Al^- ion current was low. For this reason the uncertainty is large, although the result is consistent with filter 12412. We thus believe the best present estimate for the $^{26}\text{Al}/^{10}\text{Be}$ production ratio is $3.8 \pm 0.6 \times 10^{-3}$. This value is lower than (but within the estimated uncertainties) those ($5.2\text{--}6.7 \times 10^{-3}$) deduced by Reyss *et al.*¹⁴, on the basis of their own measurements in sea sediments and Mn nodules, and earlier measurements in polar ice¹⁵. However, for the measurements in sediments and Mn nodules, in addition to the ^{26}Al count rate being near their limit of detection, there is as yet no assurance that the ^{10}Be and ^{26}Al will be incorporated with equal efficiencies. In fact we have measured ^{26}Al in two Mn nodules and shown that, at least in these cases, ^{26}Al and ^{10}Be were not incorporated with their production ratio (in preparation).

A ratio of $^{26}\text{Al}/^{10}\text{Be} = 3.8 \times 10^{-3}$ is also lower than the most recent theoretically calculated production ratio (5.2×10^{-3}) of Reyss *et al.*¹⁴. However, as noted by Raisbeck and Yiou¹⁶, this calculated ratio is based on proton cross-sections for ^{10}Be production, whereas neutron induced reactions are most important in the atmosphere.

While the measurements presented here represent a significant improvement in sensitivity compared with earlier accelerator results^{5–8}, the anticipated counting rate (≤ 0.1 c.p.m.) for a marine sediment (expected to have an initial $^{26}\text{Al}/^{27}\text{Al}$ ratio of $\sim 10^{-14}$) would still make direct measurements only marginally practical. It is possible that an isotope enrichment technique¹⁷ will still be necessary for measurements of this type, and we are working on such a procedure.

^{26}Al has been used for some time in studies of meteorites and lunar material. The much larger concentration of ^{26}Al in these extraterrestrial materials means that it can be measured by the radioactivity decay technique, although only using long counting times and quite large (≥ 1 g) samples. The only measurements of ^{26}Al in extraterrestrial matter using an

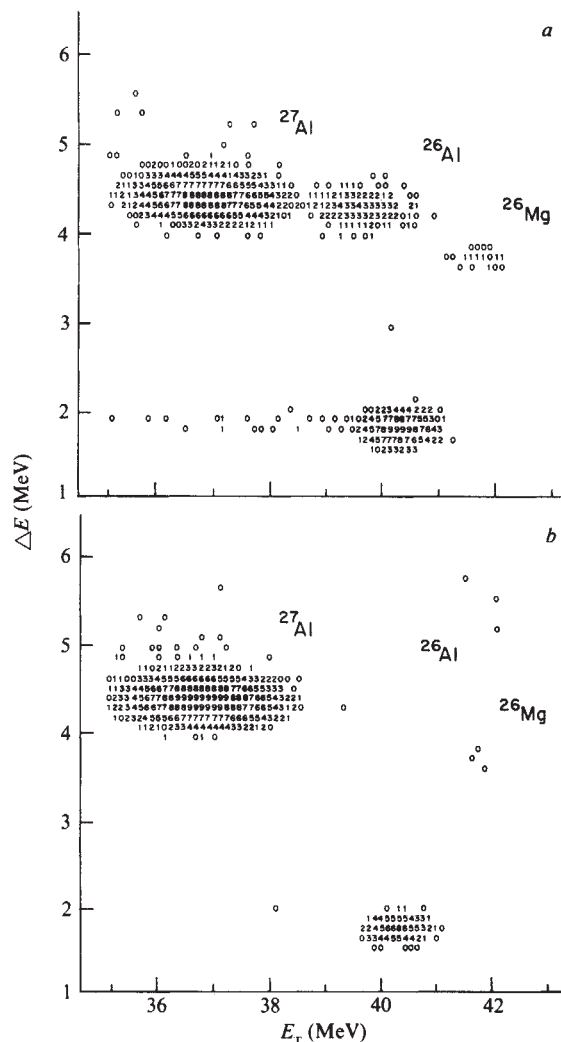


Fig. 1 Particle identification spectra where energy loss (ΔE) is plotted against total energy (E) for: *a*, lunar sample 61241; and *b*, reagent Al_2O_3 . The numbers (n) in the spectra indicate the number of events (N) through the relationship $2^n \leq N < 2^{n+1}$. Total accumulation times were 14 and 16 min for *a* and *b* respectively. The total number of ^{26}Al events in *a* is 375.

accelerator that we are aware of are by Thomas *et al.*⁸ on ~ 1 g samples of iron meteorite. The great sensitivity available with our technique means that measurements of ^{26}Al in very small quantities of extraterrestrial material are feasible in a period of the order of 1 h. To demonstrate this we made measurements on two lunar soil samples of ~ 2 mg each (in fact, only about one quarter of each sample was actually used in the accelerator measurement). These experiments are being made in cooperation with the planetology group at the Laboratoire René

Bernas as a means of studying the irradiation history of lunar soils. The samples were dissolved in HF, together with 1 mg of Be and 2 mg of Al carrier. As before, the Be and Al were extracted by acetylacetone, and then separated on an ion exchange column. A spectrum from one of the measurements, together with one from a blank Al_2O_3 sample, is shown in Fig. 1. The results are given in Table 1. A previous measurement on a much larger sample of surface soil 61241 gave 183 ± 7 d.p.m. kg^{-1} sample¹⁸ which is within 8% of our value (169 ± 22 d.p.m. kg^{-1}) when translated into the same units. To our knowledge no other ^{26}Al measurement is available for soil 61221. While our value seems high for a sample of 30–35 cm depth, this particular sample has been found to be anomalous in many other respects^{19,20}. Further measurements will be necessary to assess the significance of the ^{26}Al result in this case. Despite the high ^{26}Al concentration, the $^{26}\text{Al}/^{10}\text{Be}$ ratio in the deep sample is, as expected, lower than in the surface sample. This is due to low-energy solar protons having larger cross-sections for ^{26}Al production than ^{10}Be .

One dramatic effect evident in Table 1 is the very large difference between the $^{26}\text{Al}/^{10}\text{Be}$ ratio produced in the atmosphere and extraterrestrial environments (this comes about basically because the only significant progenitor for ^{26}Al in the atmosphere is the $\sim 1\%$ of argon). With an improvement on the present upper limit for ^{26}Al in tektites²¹, the $^{26}\text{Al}/^{10}\text{Be}$ ratio could be a useful parameter for distinguishing between a terrestrial or extraterrestrial origin for the ^{10}Be recently found in tektites²².

As can be seen from Fig. 1, the amount of lunar sample used is still a long way from the minimum quantity needed. In fact, based on our observed background, and a reasonable measuring time, it should be possible to make measurements (to $\pm 15\%$) on extraterrestrial samples at least 20 times smaller that is ~ 100 μg . This offers many potential new applications of ^{26}Al , as, for example, measuring it in individual sand sized lunar grains, high spatial resolution studies in meteorites, and detection in various forms of other extraterrestrial material. We have measured ^{26}Al in both iron and silicate 'cosmic spherules', and the results will be presented elsewhere.

Although all the ^{26}Al results presented here have been calculated relative to a standard, it is also possible with the accelerator technique to obtain an absolute value for the $^{26}\text{Al}/^{27}\text{Al}$ ratio in the sample. If such a measurement is made on a sample whose absolute radioactive disintegration rate is known, an independent check of the half life can be made. Because several authors have raised questions regarding the reliability of the presently accepted ^{26}Al half life^{23,24} we carried out a special run to measure the absolute ratio of both the Saclay standard, and one prepared from an ^{26}Al standard obtained from the National Bureau of Standards. The $^{26}\text{Al}/^{27}\text{Al}$ ratio in the sample prepared from the NBS standard was sufficiently concentrated ($\sim 1 \times 10^{-8}$) that the accelerator could actually be tuned on the ^{26}Al count rate. (The cross-contamination from the NBS standard necessitated a complete cleaning of the ion source before further measurements on natural samples could be made.) The details of this experiment will be presented elsewhere. The results with the two standards were in excellent agreement with each other, giving effective half lives of 680,000 and 710,000 yr for the Saclay and NBS standards respectively, with estimated uncertainties of $\sim 10\%$. We thus feel it is unlikely that there is any substantial error in the presently accepted half life of $716,000 \pm 32,000$ yr (refs 25, 26).

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Catalytic activity of freshly generated chromium electrode surfaces

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We report here the enormous acceleration of the hydrogen evolution reaction on chromium which we have observed when the metal surface is freshly generated at low electrode potentials in aqueous solutions. It is quite remarkable that this reaction continues to occur at a very fast rate long after the metal surface has been created provided the potential remains low.

The technique which we use to generate electrode surfaces in aqueous electrolytes while maintaining control of the electrode potential has been described previously^{1–3}. The new surface is created mechanically by allowing a diamond stylus to impinge on the surface of a rapidly rotating (100 Hz) disk electrode for a period of ~ 1 ms. This machining process is performed when a steady state current has been achieved on the electrode as a whole by maintaining a constant electrode potential using a fast response potentiostat. The current response of the electrode consequent on generating the scratch surface is then measured using a series of transient recorders set up so as to record the initial and longer term effects as well as the pre-trigger behaviour. The scratch scar appears as an arc of ~ 2 mm length, 26 μm width and ~ 2 μm depth. Results reported here were achieved in a de-aerated 1.0 M KOH aqueous electrolyte solution at a temperature of 292 ± 2 K. By appropriate control of the electrode potential both anodic and cathodic events may be recorded, although here we consider only the hydrogen evolution process.

One such cathodic current transient due to a scratch generated on pure chromium in 1.0 M KOH at a potential E , of $-1,305$ mV (nhe) is shown in Fig. 1. This potential is more negative than the equilibrium potential for reversible hydrogen evolution (-827 mV(nhe) at pH = 14, 1 atmosphere of hydrogen). For times earlier than stylus contact Fig. 1 shows the steady state current I_0 , flowing from the electrode as a whole. The rise in cathodic current due to the scratch occurs for the period of stylus contact, t_c , and represents excess current flowing from the scratch surface. By measuring the maximum rate of current increase the maximum current density on the freshly bared metal surface can be calculated as $i_s = (2\pi r \omega \gamma)^{-1} dI/dt$, where dI/dt is the rate of increase of current, ω the electrode rotation rate, γ the scratch width and r is its distance from the centre of rotation. After stylus contact has been broken, the

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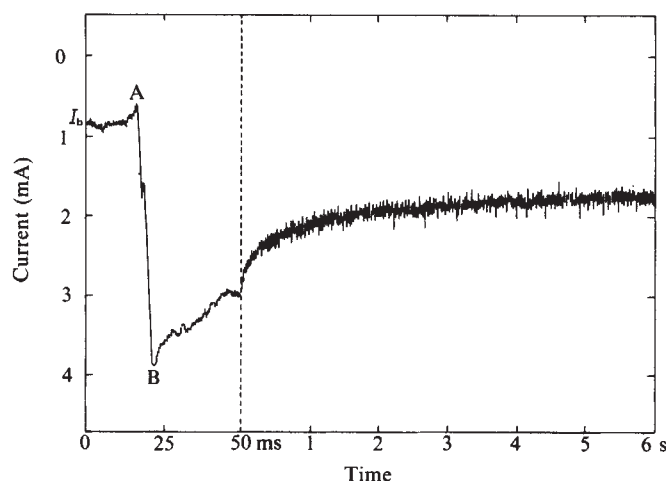


Fig. 1 Cathodic current transient due to generating a scratch on pure chromium at a potential of $-1,305$ mV(nhe) in 1.0 M KOH. Note the change in time scale and the incomplete decay of current to the base level. A, scratch commencement; B, scratch completion.

scratch is complete and the current decays with time; this is shown in Fig. 1 for up to 6 s. The current density on the scratch in this region may also be quantified by $i_s(t) = (2\pi\omega\gamma t_c)^{-1}(I(t) - I_b)$ where $I(t)$ is the total current flowing from the whole electrode at time t after scratching.

The remarkable feature of Fig. 1 is that the cathodic current density on the scratch does not decay to the original steady state. Instead, a new steady state current density, $i_s(\infty)$, is established, which exists indefinitely, provided the electrode potential is maintained. Because the only electrochemically reducible species in solution is water the entire cathodic transient represents an accelerated evolution of hydrogen from H_2O . Values of i_s and $i_s(\infty)$ are shown as a function of potential in Fig. 2, together with the steady-state current density i_b which flows through an unscratched chromium electrode. Figure 2 shows that the enormous cathodic acceleration is achieved over a range of potentials. The data have been corrected for the ohmic potential drop to the scratch³; the correction is, however, small and is only necessary for $i_s(\infty) > 3$ kA m^{-2} . Both i_s and $i_s(\infty)$ obey Tafel's law with cathodic Tafel slopes $\partial E / \partial \log i_s = -156$ mV and $\partial E / \partial \log i_s(\infty) = -142$ mV. These Tafel slopes may be interpreted mechanistically as rate determining electron transfer, that is $\text{H}_2\text{O} + e^- \rightarrow \text{H} + \text{OH}^-$, where H is a hydrogen atom adsorbed to the scratch surface. Recombination of the H atoms thereby produced must therefore be fast.

Extrapolation of the new steady state current density $i_s(\infty)$ to the equilibrium potential for hydrogen evolution gives an exchange current density of 0.4 A m^{-2} . The value is very high indeed, and is comparable with hydrogen exchange current densities of such catalytic surfaces as Pd in similar solutions^{4,5}.

This spectacular rate of hydrogen evolution on a metal conventionally thought to be a poor hydrogen catalyst can be observed visually by halting the electrode rotation after scratching. Under these circumstances the scratched area is seen to be decorated with a necklace of H_2 bubbles as in Fig. 3. Although the entire electrode surface is evolving H_2 , the density of bubbles on the scratch is clearly very much greater than that on the unscratched area.

The initial acceleration of the hydrogen evolution reaction giving rise to the cathodic current density i_s , is clearly associated with removal of the surface oxide film during scratching. The fact that the steady state achieved after scratching, $i_s(\infty)$, is different from that observed originally means that the original oxide film is not reformed. Clearly, the oxide film cannot be completely electrochemically reduced, even at potentials where this reduction is thermodynamically possible⁶. That decay in cathodic current which does occur can be due to two possible

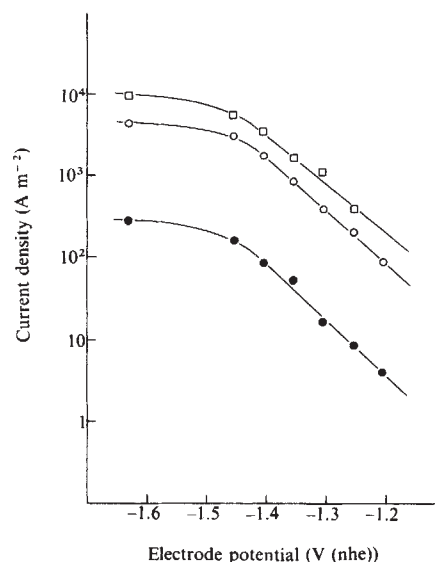


Fig. 2 Cathodic current density on chromium as a function of potential. □, Instantaneous scratch current density i_s . ○, Steady state scratch current density $i_s(\infty)$. ●, Steady state current density on unscratched chromium i_b .

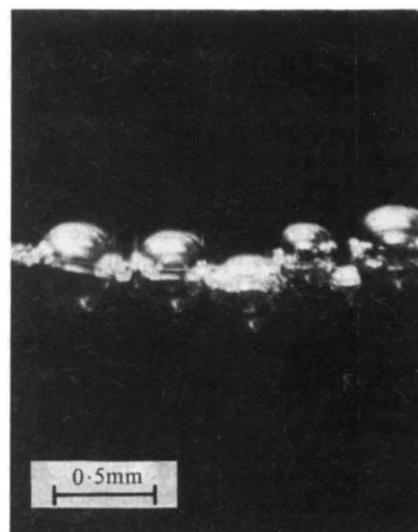


Fig. 3 Photomicrograph of H_2 bubbles evolved from the scratched area of a pure chromium electrode under potentiostatic control at $-1,255$ mV(nhe) in 1.0 M KOH. Picture taken ~ 200 s after scratching.

reasons: (1) the partial reformation of an oxide film, or (2) the growth of hydrogen bubbles causing blockage of the scratch surface. Reformation of oxide film can occur at potentials lower than the thermodynamic equilibrium potentials for the bulk chromium oxides⁶, as is shown by the potential range covered in Fig. 2. In either case, however, the results demonstrate the powerful and permanent catalytic activity of the new chromium surface towards the hydrogen reaction.

This type of behaviour has not before been reported for mechanically generated electrode surfaces reacting with water, despite the long history of examination of such processes⁷⁻⁹. Enhanced catalytic activity of platinum after extensive cathodic pretreatment in acid solutions¹⁰ has been ascribed to removal of the oxide film although the presence of anions was thought to interfere with the interfacial electrode processes. Similar phenomena have been described¹¹ after ultrasonic treatment of the platinum to remove the oxide film. This work provides unambiguous evidence of such enhanced activity due to oxide film removal, because the only available anodic reaction is the

oxidation of chromium by water. It also demonstrates the existence of some type of oxide film at very low potentials.

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Electrical conductivity in phthalocyanines modulated by circularly polarized light

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The phthalocyanines, both metal-free and metal-substituted (Fig. 1), are well known as gas absorbers and useful as ambient vapour sensors^{1–5}. The increase in conductivity which occurs on irradiating phthalocyanine films with light (the photovoltaic effect) is a relatively slow diffusion-controlled process^{6–9}. We report here the modulation of electrical conductance by circularly, as opposed to linearly, polarized light of phthalocyanine films sublimed onto an interdigital electrode surface [metal-semiconductor-metal (MSM) structure]. The influence of an adsorbed electron acceptor, the oxygen molecule, in creating the p-type semiconductor has been investigated. The underlying mechanism of the conductance change involves change of the Fermi level of the phthalocyanine electrode system by circularly polarized light acting as an effective magnetic field in an inverse Faraday effect.

Light can interact with the phthalocyanine films in an electron-accepting atmosphere in two different ways. Either a sharp resonance interaction can occur with the electron acceptor bound to the metal^{10–15}, or a broad resonance with the electron acceptor adsorbed to the surrounding phthalocyanine ring. Both metal-free and copper-substituted phthalocyanine films were studied, and both exhibited circularly polarized light-induced modulation effects, although the resistances of the metal-free films were modulated to a greater extent (~16%) than were the copper-substituted (~5%).

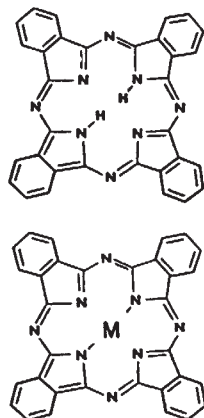


Fig. 1 Metal-free phthalocyanine (H₂Pc) structure, top, and metal-substituted phthalocyanine (MPc), bottom.

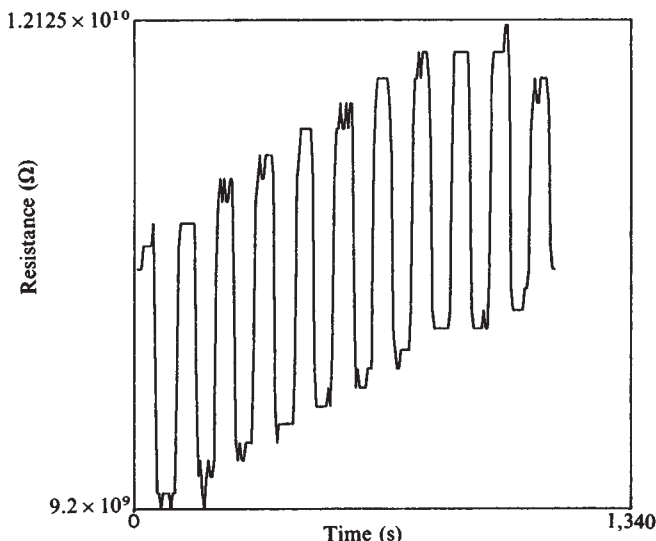


Fig. 2 Light-induced modulation of an H₂Pc film conductivity. Abscissa, a temporal interval of total length 2,500 s (250 counts each integrated over 10 s). Increasing conductivity is in the downward direction. The lasing line used = 4,765 Å of intensity 15 mW. The voltage bias on the MSM structure was 0.5 V. The light was changed in polarization from left to right, then left, etc. circularly polarized, in periodic intervals.

The preparation of the MSM structure (the phthalocyanine films becoming p-type semiconductors on exposure to oxygen or an electron acceptor) involved the sublimation of purified copper-substituted phthalocyanine, CuPc, in one case, and metal-free phthalocyanine, H₂Pc, in another case, on interdigital electrode surfaces consisting of 50 pairs of gold 'fingers' (2,000 Å Au on 200 Å Cr). Each finger was 25 μm wide and the space between each finger was 25 μm. The fingers had an overlap distance of 7,250 μm. The average resistance of a clean, dry, microelectrode was $>3 \times 10^{11} \Omega$, which allowed films with surface resistivities of $<10^{15}$ per square to be easily studied. The light intensity was measured at all polarizations and found to be the same.

A typical modulation profile over time is shown in Fig. 2, in which the laser irradiating the H₂Pc film is changed periodically from left to right circular polarization. As the incident left circularly polarized light is expected to deplete the density of hole (p) carriers, the effect is describable by a modification of the well known equation for hole densities:

$$p = (N_v - \eta) \exp \frac{(-E_F - E_v - \xi)}{kT}$$

where E_F is the Fermi energy level; E_v , the energy of the valence band; N_v , the density of states in the valence band; ξ , a variable dependent on ambient gas absorption and bias voltage; and η , a variable dependent on the number of photons of incident circularly polarized light.

This description is in agreement with recent evidence that the applied field and current density influence the position of the Fermi level of a phthalocyanine film¹⁶, the level achieved being the quasi-Fermi level.

Other observations are: (1) circularly polarized light can either increase or decrease the electrical conductivity of the phthalocyanine films depending on the magnitude of the Fermi level which is set by both the ambient atmosphere and the bias voltage of the MSM structure. This result agrees with previous reports^{17–21}, and counters any suggestion that the modulation effect is due to intensity differences in the polarizations, despite such differences being undetected by photon counting equipment. (2) The interaction of circularly polarized light and phthalocyanine films shows a polarization preference: left circularly polarized light has an antagonistic action to right circularly polarized light. (3) The photoconductivity (photovoltaic

effect) and the polarized light modulation of conductivity can be abolished by the immersion of the MSM structure in N_2 , He or Ar gas, indicating the requirement of an electron acceptor, such as O_2 , for both effects.

The circularly polarized light-induced modulation of the resistance of the MSM structures, observed in these studies, is due to the removal by light interaction of the electron-accepting oxygen molecule. The absorbed oxygen probably spin pairs with an electron from the phthalocyanine ring and the circularly polarized light, acting as an effective magnetic field, populates magnetic substates which precludes this interaction.

An inverse Faraday effect²²⁻²⁴, in which circularly polarized light induces paramagnetism in an irradiated sample, is a possible mechanism mediating this magnetic substate population. On return of the paired electrons to the phthalocyanine ring, the hole carriers would be removed. Such an inverse Faraday effect exists in the porphyrins²⁵⁻²⁷—a species of biological molecule closely related to the phthalocyanines. The paramagnetic switching of phthalocyanine films in an inverse Faraday effect²⁸ also indicates a possible use of these films in future computer technology.

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Growth history of hydrothermal black smoker chimneys

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Hollow mineral spires known as 'black smoker' chimneys precipitate around 350 °C jets of hydrothermal fluid spouting from the sea floor on the East Pacific Rise (EPR) at 21° N (refs 1, 2). I describe and discuss here the formation of mineral zoning across the walls of these structures throughout two major growth phases, a sulphate-dominated stage and a sulphide replacement stage. During the sulphide replacement stage, at least four distinct sulphide mineral zonation sequences develop across chimney walls from interior to exterior. The apparently successive formation of these sulphide zonation sequences is attributed to evolution of radial thermal and chemical gradients accompanying the gradual thickening and sealing of black smoker chimney walls.

Black smokers are the sooty plumes of fine pyrrhotite, sphalerite, lesser pyrite and traces of cubanite which precipitate

as superheated solutions emerge from 'chimneys' in the ocean floor and mix with seawater^{1,3,4}. Samples of three chimneys collected from black smoker vents with the *Alvin* submersible during the 1979 RISE expedition to the EPR axis at 21° N (refs 1, 3, 5), are considered in the present study. The measured temperature of the fluids flowing through the sampled chimneys was ~350 °C (refs 1, 6, 4). These fluids are highly acidic (pH ~ 4) and reducing⁴. One of the chimney samples (914 R-8) was collected ~1 m above the base of a decapitated chimney, and the other two samples (917 R-4 and 921 R-5) are tops of chimneys. Numerous polished thin sections from the three chimney samples were examined microscopically to determine the mineral content and textural characteristics of black smoker chimney walls. Analyses of the same sections were made with the electron microprobe to determine accurately specific mineral compositions and to document compositional zoning within individual grains and across chimney walls from interior to exterior.

Black smoker chimney walls typically exhibit inner zones of massive Cu-Fe sulphide enclosed within outer zones of disseminated sulphide in an anhydrite matrix (Fig. 1). Anhydrite ($CaSO_4$) in black smokers is known to be derived from seawater on the basis of sulphur isotopic evidence^{1,7}. The outermost skin of black smokers bears fine-grained pyrrhotite ($Fe_{1-x}S$), pyrite (FeS_2) and sphalerite ($Zn(Fe)S$) with textures matching those in mineralogically similar black plume particulate assemblages⁸. Within chimney walls, a pyrrhotite-free sulphide assemblage of coarser, more euhedral $Zn(Fe)S$ (sphalerite and/or wurtzite), FeS_2 (pyrite and/or marcasite) and various Cu-bearing sulphides prevails^{3,7,9}. These sulphides often replace anhydrite outward along crystallographic planes in the sulphate.

The configuration described above suggests that chimney walls are initially constructed upwards from the sea floor by anhydrite precipitation from seawater which is heated to supersaturation with respect to $CaSO_4$ around the margins of a discharging jet of hydrothermal fluid. This nascent phase of sulphate-dominated chimney growth, designated as 'stage I', is depicted in Fig. 1. In the outer skin of the chimney, hydrothermal fluid which is trapped as microscopic inclusions within anhydrite or which leaks out into the exterior by dissolution of anhydrite along cleavage planes is rapidly cooled and precipitates the fine-grained, pyrrhotite-bearing assemblage resembling black smoke particulates. Throughout the hot, active life of the chimney, anhydrite continues to build chimney walls upwards and outwards at the interface between the chimney and seawater. Metastable pyrrhotite precipitated at the chimney exterior is dissolved and reprecipitated as pyrite (or marcasite) when the exterior skin becomes incorporated within the chimney wall by the outward accretion of anhydrite. Pyrite thus is ultimately the most abundant iron-sulphide in black smoker chimneys.

Once the anhydrite walls of black smokers are in place, hydrothermal fluid is protected from extensive mixing with seawater and Cu-Fe sulphides begin to grow into the chimney centre. This introduces 'stage II' (Fig. 1), which is dominated by sulphide precipitation. During stage II, hydrothermal fluids migrating outward from the chimney centre dissolve and replace anhydrite with sulphide minerals. The chimney at this point is growing upwards (by precipitation of anhydrite), inwards (by precipitation of Cu-Fe sulphides) and outwards (by anhydrite accretion and subsequent replacement of anhydrite by sulphides).

Four different sulphide zonation sequences are known to precipitate during stage II. These sequences, shown in Fig. 2, are divided into one bornite-bearing (bn) sequence and three bornite-absent sequences. The bn sequence has a central zone of chalcopyrite ($CuFeS_2$) enclosed within an outer envelope of continuously-zoned bornite-chalcocite solid solution (Cu_5FeS_4 – Cu_2S). The bornite envelope grades outward from anomalous¹⁰ sulphur- and iron-rich compositions at the chalcopyrite contact through normal bornite exhibiting an outward decline in iron content (pinkish in reflected light) to iron-poor bornite (bluish-

grey) or chalcocite (bluish-white) in the outer chimney wall. Bornite–chalcocite solid solution (ss) in chimney exteriors is associated with covellite (CuS), digenite (Cu_9S_5), and with previously described Fe- and Zn-sulphides. Cu/Fe atomic ratios across the chalcopyrite and bornite zones of the bn sequence increase consistently from interior to exterior (Fig. 2).

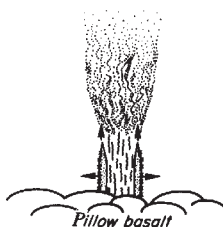
Bornite-absent sequences include the chalcopyrite-dominated (cp) sequence, the chalcopyrite–intermediate solid solution (cp–iss) sequence and the cubanite (cb) sequence. The chalcopyrite-dominated sequence has a central chalcopyrite zone with a smooth, outward-increasing Cu/Fe variation exactly like that of the bornite-bearing sequence (Fig. 2a); however, the outer zone of the cp sequence contains Cu–Fe sulphide grains randomly varying in composition from Fe-rich chalcopyrite to Fe-rich iss to cubanite (CuFe_2S_3). In the cp–iss sequence, Cu/Fe ratios in the outer margin of the central chalcopyrite zone reverse to an outward-decreasing trend (Fig. 2b), and fine, dark lamellae of iss begin to exsolve from Fe-rich cp at the exterior edge of the central cp zone. The abundance of the iss lamellae in these ‘mixed’ cp–iss grains increases towards the chimney exterior, resulting in a systematic outward decrease of Cu/Fe ratios across the outer chimney wall of the cp–iss sequence. The cubanite sequence is characterized by the absence of chalcopyrite. The central zone consists of isotropic, Fe-rich iss with a composition and an X-ray diffraction pattern matching that of cubic (high temperature) cubanite¹¹. Rare pyrrhotite is occasionally intergrown with cubanite in the central zone. Because cubanite is also the only Cu–Fe sulphide present in the outer zone of the cb sequence, the Cu/Fe ratio remains constant across the entire cb sequence (Fig. 2c). The compositions of Cu–Fe sulphides in bornite-absent sequences are plotted in Fig. 3 on a blow-up of the central region in a Cu–Fe–S triangular phase diagram constructed at 350 °C by Sugaki *et al.*¹². The arrows in Fig. 3 trace the reversal of the Cu/Fe ratio trend observed from interior to exterior across the cp–iss sequence. The compositions of chalcopyrite (light) and iss (dark) lamellae are plotted as stars, and the scatter of points between the stars represents the bulk compositions of finely-exsolved mixed grains with different proportions of chalcopyrite and iss. Cubanite compositions are shown on Fig. 3.

Sulphide/sulphate ratios, which are a measure of the extent of sulphate replacement by sulphide, and total Zn concentrations vary among the different zonation sequences. Lower sulphide/sulphate ratios and total Zn are observed in the bn-bearing and cp-dominated sequences relative to the cp–iss and cb sequences because the latter show greater replacement of outer zone anhydrite by $\text{Zn(Fe)S} + \text{FeS}_2$ assemblages.

Sulphide zoning in chimneys is a dynamic phenomenon featuring spatial shifts and oscillations of zone boundaries within chimney walls and transformations from one zone sequence to another with time. Radial expansions and contractions of mineral zones are recorded in individual sulphide grains as replacement textures, exsolution, growth-banding, overgrowths, and compositional zoning.

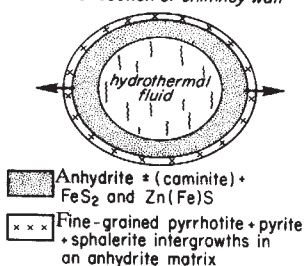
There is evidence that the bn-bearing sequence develops early in stage II and successively evolves into the cp-dominated, cp–iss, and cb zonation sequences as chimney walls mature. Early formation of the bn and cp zonation sequences is indicated by the inevitable occurrence of the bornite sequence in chimney walls <~5 mm thick and by the nominal degree of sulphate replacement by sulphide in both of these sequences. The development of the cp-dominated sequence by outward chalcopyrite and iss replacement of bornite is strongly suggested by the occurrence of remnant bornite grains in the outermost edge of the cp sequence chalcopyrite zone. The cp–iss sequence presumably inherits the Cu/Fe profile of the bn sequence chalcopyrite zone. The abundance of sulphide in the cp–iss and cubanite sequences indicates evolution of these sequences late in stage II. The cp–iss sequence has mineralogical and textural characteristics intermediate between the cp-dominated and cubanite sequences and is apparently a transitional link between these two sequences.

STAGE I



Arrows indicate directions of growth

cross-section of chimney wall



STAGE II

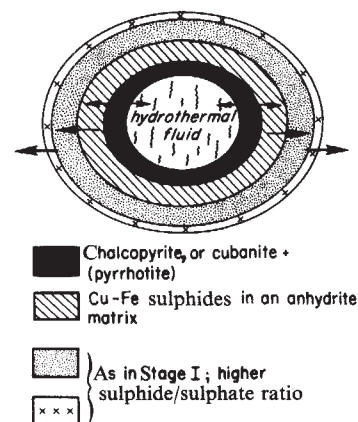
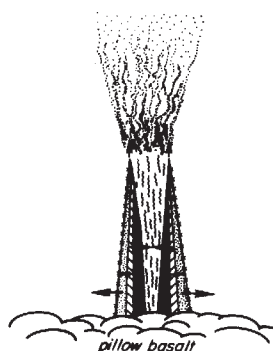


Fig. 1 First-order model of black smoker chimney growth. Stage I, chimney construction begins with the upwards and outwards accretion of seawater-derived anhydrite walls. A new Mg-hydroxysulphate-hydrate mineral, to be named ‘caminite’³, occasionally precipitates along with anhydrite from heated seawater. The exterior skin of the chimney bears fine-grained, branching intergrowths of pyrrhotite, pyrite and sphalerite which precipitate from rapidly chilled hydrothermal fluid. This exterior sulphide assemblage is incorporated into the chimney wall by the continuous outward growth of anhydrite. Within the chimney, metastable pyrrhotite is removed by dissolution. Minor FeS_2 (pyrite and/or marcasite) and Zn(Fe)S (sphalerite and/or wurtzite) are distributed throughout the anhydrite wall, and minor silicate phases (such as amorphous silica, talc, aluminosilicate gel; not shown) may also precipitate in the outer wall of the chimney during stage I. Stage I growth continues in the peripheral zones of chimneys throughout the hot, active life of black smokers. Stage II, the formation of anhydrite walls protects hydrothermal fluids from mixing and allows Cu–Fe sulphides to precipitate within the central conduit and to replace anhydrite and other sulphides in the outer chimney wall. The sulphide/sulphate ratio of the outer chimney increases as FeS_2 and Zn(Fe)S grains grow at the expense of anhydrite.

The progressive development of sulphide zonation sequences is interpreted here as an outcome of changes in thermal and chemical conditions within chimney walls which transpire as black smokers age. Young chimney walls are thin shells of anhydrite which are subject to thermal fracturing and to interior corrosion by highly reducing hydrothermal fluids. Breaching of chimney walls by cracking and dissolution permits mixing across walls between seawater and hydrothermal fluid. Mixing sets up increasing gradients in oxygen fugacity (f_{O_2}), sulphur fugacity (f_{S_2}) and pH from interior to exterior across chimney walls, all of which are conducive to the formation of the bn sequence. The influence of f_{O_2} and f_{S_2} gradients on the formation of the bornite sequence is illustrated by the phase diagram in Fig. 4 (modified from ref. 13), which schematically depicts the suc-

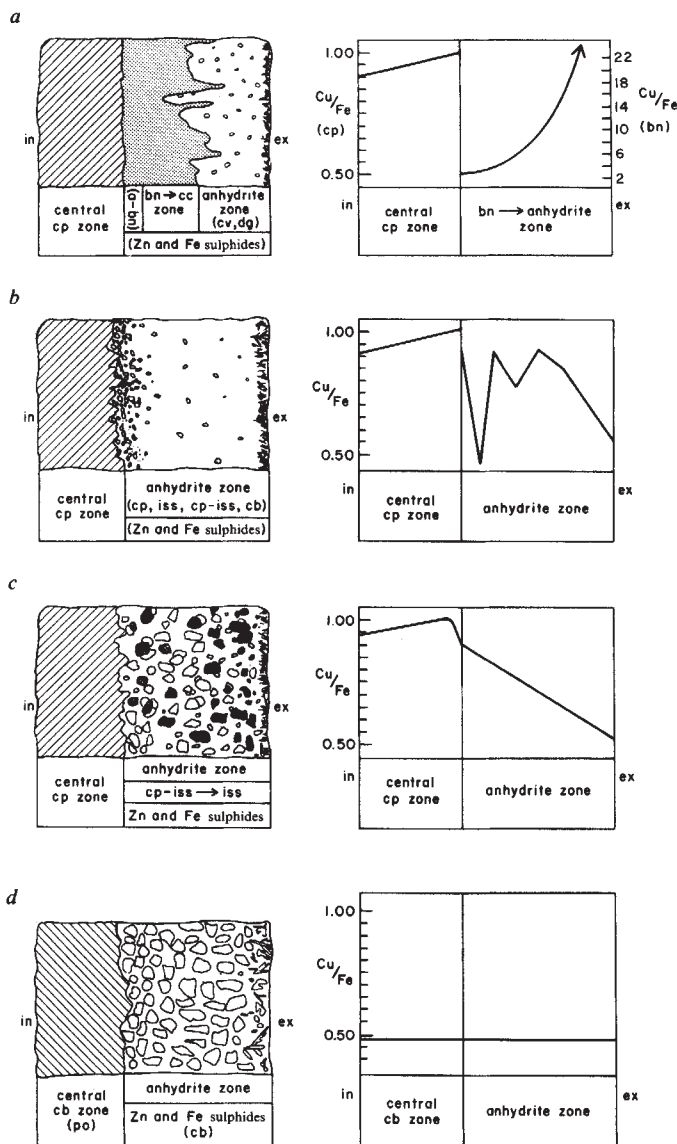


Fig. 2 Left: Four sulphide mineral zonation sequences which develop across chimney walls during stage II. Wall thickness varies from <5 mm for the bornite-bearing sequence (a) to >3 cm for bornite-absent sequences (b-d). Right: Variations of the Cu/Fe atomic ratios of Cu-Fe sulphides across the zonation sequences shown at left. Cross-hatching of mineral grains in c indicates exsolution of intermediate solid solution (dark) from chalcopyrite (light). Minor mineral components are enclosed in parentheses. in, Interior chimney wall adjacent to central conduit; ex, exterior chimney wall adjacent to seawater; cp, chalcopyrite; a-bn, anomalous bornite; bn, bornite; cc, chalcocite; cv, covellite; dg, digenite; iss, intermediate solid solution; cp-iss, chalcopyrite-intermediate solid solution 'mixed grains' (see text); cb, cubanite; po, pyrrhotite. b, Chalcopyrite-dominated sequence; c, chalcopyrite-iss sequence; d, cubanite sequence.

cession of chalcopyrite → bornite → chalcocite → covellite which is expected along a path of increasing sulphidation and oxidation. Increasing pH towards the chimney exterior enhances the efficacy of Cu-bisulphide complexing¹⁴ and thereby favours the formation of the bornite sequence by promoting the establishment of an outward-increasing gradient in dissolved Cu/Fe.

In time, anhydrite precipitation gradually thickens walls, seals voids and fractures, and checks the inward incursion of seawater. Mixing across chimneys is eventually eradicated, and, in the absence of advective cooling, chimney walls become hotter. Reducing hydrothermal fluids migrate pervasively into the outer

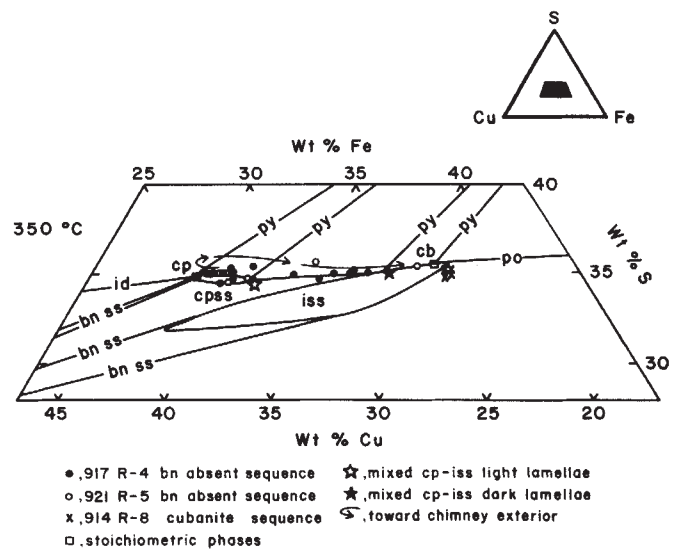


Fig. 3 Compositions of Cu-Fe sulphides in bornite-absent sequences. Electron microprobe analyses of individual grains plotted on a blow-up of the central region in a Cu-Fe-S triangular diagram constructed at 350 °C by Sugaki *et al.*¹². The arrows trace a reversal of the Cu/Fe ratio trend observed from interior to exterior across the cp-iss sequence. The scatter of points between the stars represents bulk compositions of 'mixed grains' containing different proportions of chalcopyrite and iss (see text).

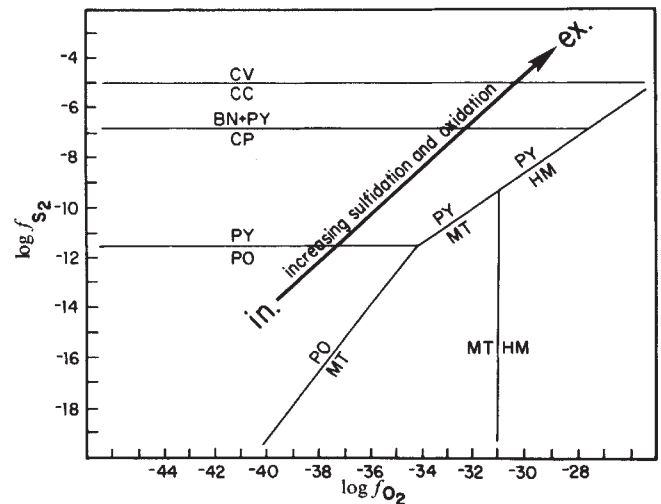


Fig. 4 $\log f_{S_2}$ - $\log f_{O_2}$ diagram at 300 °C and 500 bar, after Brimhall¹³ illustrating the sequence of sulphides which are stable along a schematic gradient representing increasing f_{S_2} and f_{O_2} from interior (in) to exterior (ex) across chimney walls. cv, covellite; cc, chalcocite; bn, bornite; py, pyrite; cp, chalcopyrite; po, pyrrhotite; mt, magnetite; hm, haematite. The progression from cp → bn → cc → cv mirrors the bornite-bearing zonation sequence in black smokers. Pyrrhotite is thought to be stable in equilibrium with unmixed, oxygen-poor hydrothermal fluid; pyrite is in equilibrium with mixed hydrothermal solutions containing a seawater component.

chimney, and bn-absent zonation sequences appear as black smoker walls re-equilibrate. It seems that in sufficiently reducing conditions the stable sulphides in chimney walls are cubanite and pyrrhotite. Because these sulphides are also present in the black smoke particulate assemblage which precipitates when 350 °C hydrothermal fluids are quenched by 2 °C seawater, it is reasonable to suggest that cubanite and pyrrhotite are in equilibrium with unmixed hydrothermal effluent.

Hydrothermal black smoker chimneys precipitating on the EPR axis at 21° N are mineralogically zoned structures with a complex, two-stage growth history. During stage I, anhydrite

precipitating from heated seawater builds walls upwards and outwards around outflowing hydrothermal fluids. Stage II witnesses the growth of Cu-Fe sulphides into the central conduit of the chimney and the replacement of pre-formed anhydrite by sulphide minerals. A bornite-bearing sulphide zonation sequence containing high sulphidation state sulphides forms initially along thermal and chemical gradients generated by mixing between seawater and hydrothermal fluid across young, permeable chimney walls. Later, as continuing anhydrite precipitation seals out seawater, the minerals of black smoker walls re-equilibrate through two transitional bornite-absent sulphide zonation sequences into a mature sequence which features cubanite as the only Cu-Fe sulphide. The cubanite sequence evidently develops at $\sim 350^\circ\text{C}$ in low f_{O_2} - f_{S_2} - $p\text{H}$ conditions maintained by sustained contact with unmixed hydrothermal fluids.

The formation of mineralized chimney structures similar to

those described here requires hot spring discharge into an aqueous Ca^{2+} - and SO_4^{2-} -enriched environment, and such structures are thus expected to occur only in high-temperature, submarine hydrothermal settings. The sulphide mineral zoning which develops across the walls of black smokers is, however, controlled by thermal and chemical gradients common to most hydrothermal environments, marine or terrestrial. Finally, we should point out the sobering implications of the prodigious growth rate of black smoker chimneys and the rapid formation of multiple generations of sulphides to those attempting to unravel the depositional histories of ancient hydrothermal systems.

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Measurements of acoustic correlation in the ocean with a high frequency echo-sounder

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Measurements in a British Columbia fjord, using a high frequency echo-sounder, have provided the basis for inferring both mean and random components of scatterer motion at ranges up to 100 m. The measurements demonstrate the potential for a novel technique of flow speed measurement using an echo-sounder and show the way in which the random component of scatterer motion decorrelates the resulting signal. The results indicate decorrelation times of several seconds, illustrating the passive behaviour of the scatterers. This behaviour makes the scatterers ideal targets for remote sensing of flow properties and represents a limiting example of the conditions in which such measurements can be made. The measurements provide a new source of information relevant to the study of zooplankton behaviour; in more active oceanographic conditions the relative motion of passive acoustic targets may also provide a basis for inferring the corresponding water motions and in particular, turbulence.

Sound in the ocean is scattered by particulates¹, biota², bubbles³ and temperature microstructure⁴ and the resulting acoustic cross-section and distribution can provide information on the nature and behaviour of the scatterers themselves. A different property of the signal which has received much less attention, but which has important implications for acoustic remote sensing in the ocean, is its correlation structure as a function of time. Decorrelation of the signal can arise through random motion of the scatterers, which may be active or may be simply a consequence of a random component in the fluid flow. If the scatterers are passive their correlation structure depends on both the mean and turbulent components of the

flow. In the absence of turbulence the scattered sound from successive transmissions will remain correlated, although the location of the correlation maximum will shift in space in proportion to the large scale movement of the scatterers relative to the sonar; it is this principle that underlies the concept of the correlation sonar⁵. Active movements such as swimming, will complicate the interpretation. The present study was designed to determine correlation properties in the limiting conditions of the deep and protected waters of a fjord.

The measurements were made from the RV *Vector* in June 1982 during calm weather in Alice Arm, British Columbia. We used both a wide-beam (22°) 50-kHz sounder pointing vertically downwards and also a narrow-beam (3°) 100-kHz sounder pointing forwards and inclined at an angle (α) of 44° to the vertical (Fig. 1). The pulse duration was 0.2 and 0.1 ms respectively and the repetition period 0.277 s. For a particle moving with constant velocity, the range r from the sonar is given by

$$r = (h^2 + u^2 t^2)^{1/2} \quad (1)$$

where h is the range of closest approach and u the particle speed. Thus the range varies hyperbolically with time t . In the experiment the speed was artificially imposed by running the ship forward in the quiet water of the fjord. The speed was

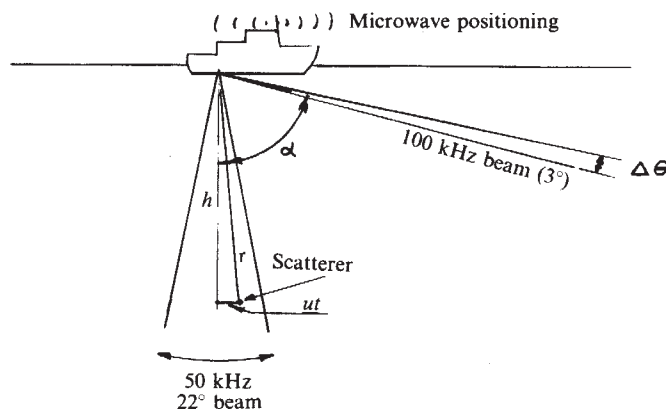


Fig. 1 Experimental arrangement incorporating a narrow beam 100 kHz echo-sounder pointing forward and a 50 kHz sounder pointing downward. In addition a range-gated Doppler sonar is mounted in parallel with the 100 kHz sounder. Navigation is carried out by microwave positioning.

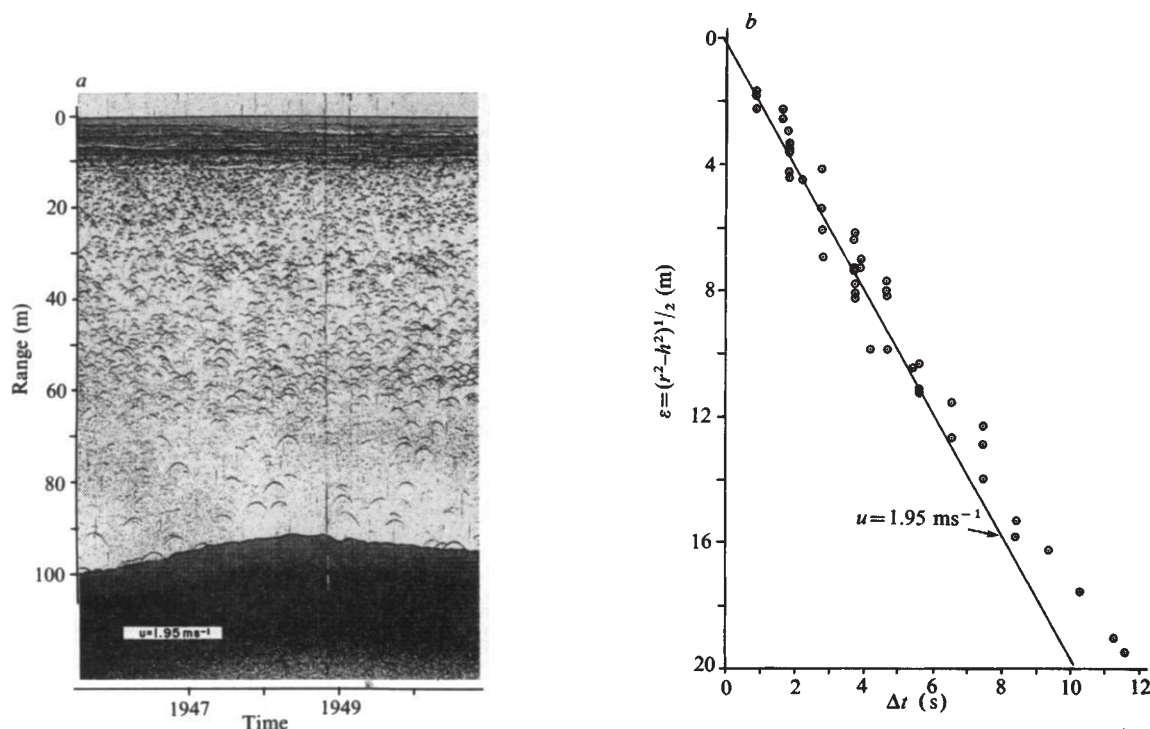


Fig. 2 *a*, Facsimile image made with wide-beam 50 kHz sonar pointing straight down with vessel moving forward at 1.95 m s^{-1} . Individual targets form hyperbolic traces as they pass through the beam. *b*, Plot of selected digitized traces from *a* between 14 and 100 m, under the transformation (2), compared with the corresponding Doppler speed measurements (solid line).

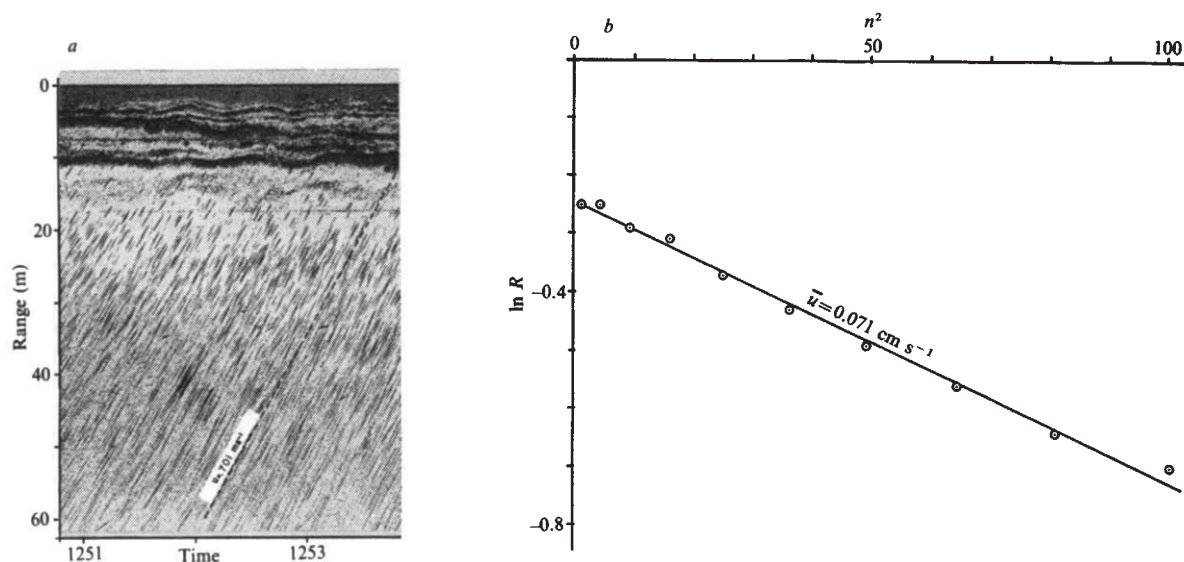


Fig. 3 *a*, Facsimile image made with narrow-beam 100 kHz sonar pointing forward at an angle of 44° to the vertical, from a ship travelling at 0.7 m s^{-1} . *b*, Cross-correlation maximum as a function of separation interval, scaled according to equation (5) where n is the number of pulse separations ($nT_r = \tau$).

independently and simultaneously determined using a range-gated Doppler sonar.

Figure 2*a* shows a facsimile recording obtained with the downwards pointing sonar. The numerous hyperbolae, with curvature inversely proportional to range, provide a sense of perspective reminiscent of the view of a pebbled beach. Figure 2*b* shows digitized points from several of the individual curves and for both positive and negative t , plotted under the range-independent transformation:

$$\xi(t) = (r^2 - h^2)^{1/2} \quad (2)$$

The estimate of flow speed perpendicular to the beam is seen to be in reasonable accord with the Doppler measurement.

For an ensemble of scatterers the acoustic signal may be described by the correlation function. The signal in our example

refers to the amplitude envelope which is detected by the echo-sounder. For scatterers having velocities with gaussian probability distribution about the mean value and standard deviation $\bar{u}$, the signal correlation $R_s(\tau)$ at lag τ can be shown⁶ to be of the form.

$$R_s(\tau) = \exp \left[-\frac{2}{3} (2\bar{u}\tau/L)^2 \right] \quad (3)$$

where L is the effective pulse length. This expression does not allow for the consequence of finite beam-width, movement of the scatterers through the beam and certain other effects which contribute to the decorrelation. It should therefore be regarded as an upper bound on the signal correlation for the relevant parameters.

The correlation properties of rapidly decorrelating scatterers cannot be resolved with our present system which has a pulse

repetition period T_r of 0.277 s. We therefore divide the scattered sound S into two portions: that which remains correlated subject only to the decorrelation effect (3) which we call the 'signal' ($\mathcal{S}$) and that portion which does not remain correlated which we call 'noise' (N), where $\mathcal{S}$ and N are normalized such that $\mathcal{S} + N = 1$. The combined correlation function $R_T(\tau)$ is then:

$$\left. \begin{aligned} R_T(\tau) &= \mathcal{S}R_s(\tau) + NR_N(\tau) & \tau > 0 \\ R_N(\tau) &= 1 & \text{for } \tau < T_r \\ &= 0 & \text{for } \tau \geq T_r \end{aligned} \right\} \quad (4)$$

From equations (3) and (4):

$$\ln R_T = \ln \mathcal{S} - \frac{2}{3}(2\bar{u}/L)^2 \tau^2, \tau \geq T_r \quad (5)$$

Thus for $\tau \geq T_r$, $\ln R_T$ should be a linear function of τ^2 . Equation (5) may thus be fitted to the data so as to determine both $\bar{u}$ and $\mathcal{S}$. The signal-to-noise ratio is then:

$$\mathcal{S}/N = \mathcal{S}/(1 - \mathcal{S}) \quad (6)$$

The cross-correlation was calculated for the amplitude envelopes S and S_r detected at the receiver between two sonar transmissions separated by an interval τ , and further subject to an axial (time) lag t' of the second profile with respect to the first. The data were integrated over a particular range gate (r_1 to r_2 from one transmission and $r_1 + t'$ to $r_2 + t'$ from the second). The time lag t' was of the order of 0–2 ms corresponding to 0–1.5 m, while the range gates r_1 – r_2 over which the calculation was made were of length 10 m. The results were further averaged over successive transmission pairs for periods of 60 s. For a particular range gate (r_1, r_2):

$$R_T(\tau, t') = \frac{\sum_{t=r_1}^{r_2} \{S(t) - \bar{S}\} \{S_r(t+t') - \bar{S}_r\}}{\left[\sum_{t=r_1}^{r_2} \{S(t) - \bar{S}\}^2 \sum_{t=r_1}^{r_2} \{S_r(t+t') - \bar{S}_r\}^2 \right]^{1/2}} \quad (7)$$

In equation (7) $\bar{S}$, $\bar{S}_r$ represents the average value of the amplitude envelope of the two detected waveforms S , S_r respectively over the range gate (r_1, r_2). As the amplitude envelope is always positive, $\bar{S}$ and $\bar{S}_r$ are non-zero. Both the maximum cross-correlation value and corresponding lag t' were evaluated from equation (7), using quadratic interpolation.

Figure 3a shows facsimile records from the narrow-beam sounder pointing forward. Targets moving through the beam yield nearly straight lines on the record, with slope proportional to $u \sin \alpha$. This slope is easily measured providing a continuous record of flow speed along the beam axis. More accurate speed estimates as a function of range were made from the observed lag for maximum cross-correlation using equation (7). The theoretical accuracy of the measurement increases in proportion to the product of pulse separation and correlation and in general it was found that this choice, typically of $\tau = 1.7$ – 2.2 s, also coincided with the minimum standard deviation in the speed estimate. For the run shown in Fig. 3a the correlation calculations yield a speed of 0.701 m s^{-1} in the depth range 60–70 m with standard deviation 0.053 m s^{-1} ; the simultaneous Doppler measurement implied a speed of 0.677 m s^{-1} . Only the amplitude envelope was digitized in this experiment. Significantly greater accuracy can be expected using phase measurements. Speeds calculated at shorter range gates were essentially identical. However at ranges < 40 m the maximum value of the cross-correlation was smaller; this must be partly a consequence of the finite beam width. At 60 m scatterers will move through the beam after 15 transmissions but at 20 m after only five transmissions.

Figure 3b shows the corresponding correlation maxima, scaled according to equation (5). The points fall close to a straight line providing satisfactory agreement with the model (4). The least squares fit yields $\bar{u} = 1.2 \text{ cm s}^{-1}$, $\mathcal{S} = 0.782$, whence $\mathcal{S}/N = 3.59$. Thus the dominant acoustic return comes

from scatterers that are essentially passive, with decorrelation times of several seconds.

Additional measurements in Saanich Inlet, a second and geographically separate British Columbian fjord, indicate comparable random motion of the scatterers. Such small values of $\bar{u}$ have implications for remote sensing in the ocean using acoustic backscatter. Measurements that depend on coherent scattering are always limited by the decorrelation time of the scatterers and a knowledge of the correlation properties is necessary for an optimum choice of sonar parameters. If decorrelation is such that the pulse separation must be less than the reverberation decay period, any pulse pair correlation is contaminated by reverberation from adjacent pulses and has a maximum value of 0.5. If the scatterers remain correlated beyond this period as in the present example, larger correlations and consequently more accurate measurements can be achieved.

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Global production of methane by termites

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Widespread destruction of forests and their replacement by grasslands may provide suitable habitats for termites, leading to an increase in their population and thus also in the production of methane¹. We have carried out an experiment to verify the role of termites in the global methane cycle and to identify the uncertainties inherent in such global extrapolations of laboratory data. We found that termites are indeed a potentially significant source of atmospheric methane with an estimated global production of about $50 \times 10^{12} \text{ g yr}^{-1}$; however, the uncertainties in global estimates are so large (10 – $100 \times 10^{12} \text{ g yr}^{-1}$) that it cannot yet be proved that termites play an important role in the global methane cycle. Our calculations show that the production of CH_4 by termites is probably $< 15\%$ of the global yearly emissions.

There is considerable evidence that the global concentration of atmospheric methane (CH_4) is slowly but steadily increasing at a rate between 1% and $2\% \text{ yr}^{-1}$ (refs 2–5). It is likely that this imbalance is caused indirectly by the rise of human population, including growth of industry and agriculture. Increase of CH_4 can lead to global warming and climatic change; it may ultimately deplete OH radicals in the troposphere, affect the Earth's stratospheric ozone layer, and increase CO and O_3 in the clean troposphere. Not enough is known about the global cycle of methane to exclude the existence of sizeable undiscovered sources and sinks. According to Peakin and Josens⁶, the production of methane by termites was first suggested by Cook as far back as 1932. Later Breznak and other investigators verified and quantified the phenomenon⁷. More recently, experiments by us and Zimmerman *et al.*^{1,8} have verified that termites produce large quantities of CH_4 . Zimmerman *et al.*^{1,8} have taken a further step and estimated that all the world's

termites together produce $150 \times 10^{12} \text{ g yr}^{-1}$ of CH_4 or up to 40% of the global production from all sources^{1,8,9}. It is primarily the large world population of termites coupled with their ability to produce CH_4 which makes them of special interest to atmospheric chemistry.

We adopted a simple but sensitive experimental procedure. A small colony of *Zootermopsis angusticollis* (Hagen) consisting of 25 workers and a queen was placed in a 0.96-l glass jar. The termites were transferred along with the piece of the damp wood that they were living in and kept at ambient temperature throughout the experiment. A part of their habitat, including galleries in the wood, was thus transferred to the laboratory. Four other similar colonies were prepared and studied. These four colonies were placed in damp filter paper, but their production of CH_4 was the same as the original colony living in its natural habitat of wood. A perforated top was placed on the jar and the wood kept moist. Two identical jars were prepared as controls. One was empty and the other contained damp wood very similar to that in the experimental (termite) jar but without termites. After a few days, the measurements of methane production by termites began. First the jar was aired, then it was sealed with an air-tight Mason jar cap. The top has a Swagelok fitting with a septum. 30–50 ml of zero air was drawn into a syringe and injected into the jar, giving it a slight positive pressure. After mixing the air, the same amount of sample, 30–50 ml was drawn from the jar. The concentration of CH_4 was measured using a gas chromatograph with a flame ionization detector (GC-FID)². The concentrations determined soon after the jar was sealed were generally close to ambient levels ($\sim 1.6 \text{ p.p.m.v.}$). We then waited for some time, typically 2–4 h, keeping the jar sealed, and sampled and analysed the air again by the same procedure as described above. The production of CH_4 was then calculated according to the following formula:

$$p(\mu\text{g CH}_4 \text{ per termite per day}) = \frac{\epsilon V \rho M C_f - C_i}{n N_0 T} \quad (1)$$

where C is the initial concentration of CH_4 (p.p.m.v.); C_f , the final concentration; n , the number of termites; N_0 , Avogadro's number; M , molecular weight (of CH_4); ρ , the density of air (molecules cm^{-3}); V the volume of jar; ϵ , the fraction of jar containing air (as opposed to wood); T , the time between the initial and final measurements (days).

The same procedures were repeated with the control jars which were sealed at the same time as the experimental jar and kept sealed for the same length of time. The results of the experiment are shown in Table 1. Emission rates of 0.8–1.0 μg per day per termite were found. On average these rates are about twice as high as those found by Zimmerman *et al.*⁸.

The extrapolation of laboratory data to the global production of methane by termites is confounded by several large uncertainties. Our approach was to estimate global production directly by the following formula:

$$P_G = \bar{p} N_\infty \quad (2)$$

where P_G is the global yearly production, $\bar{p}$ is the average production of CH_4 per termite per year (equation (1)), and N_∞ is the world population of termites. We assumed that the average production measured in the laboratory was equal to the world average. Neither $\bar{p}$ nor N_∞ is accurately known, and there are probably severe limits to the accuracy with which these quantities can be ever be known.

There is evidence of considerable natural variability of $\bar{p}$ (production of CH_4 per termite per day), both within species and among different species, and may even depend on the ecological region where termites live and on seasons⁶. The disagreement between our measurements and those of Zimmerman *et al.* may be due to the different species observed. Even more uncertain are the estimates of the world population of termites (N_∞). Experimental data reviewed by Wood and Sands¹⁰ were extrapolated by Zimmerman *et al.*⁸ to estimate that there are 2.4×10^{17} termites in the world. This number may be high as densities are generally measured where there

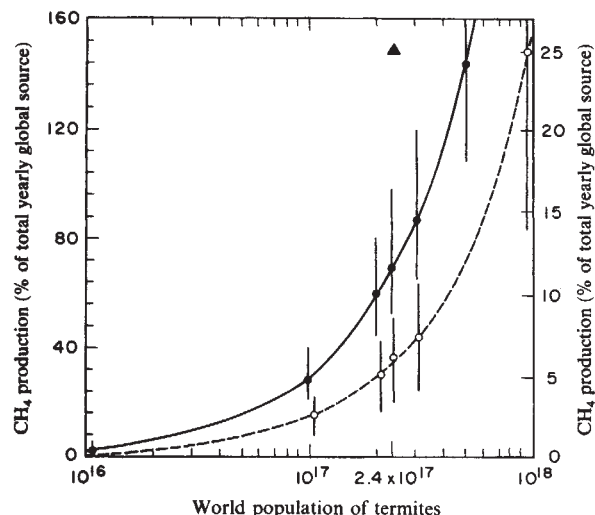


Fig. 1 The production of methane (CH_4) by termites. The solid line is based on the results of this study in which each termite produced about $0.8 \mu\text{g CH}_4 \text{ day}^{-1}$. The broken line is an analogous calculation based on similar measurements made by Zimmerman *et al.* The left vertical axis shows global CH_4 production, and the right vertical axis shows it as % of the total yearly global production of CH_4 , here taken to be about $6 \times 10^{14} \text{ g}$. ▲, Zimmerman *et al.*'s ref. 8, calculation of the global CH_4 production by termites. The vertical bars show estimates of global production based on the maximum and minimum emissions found in the two laboratory studies of CH_4 production by termites. The uncertainty becomes larger as the assumed world population of termites increases. Based on our results, we estimate CH_4 production by termites to be between 10 and $90 \times 10^{12} \text{ g yr}^{-1}$ or 2–15% of the global emissions.

Table 1 Production of methane (CH_4) by termites [*Zootermopsis angusticollis* (Hagen)] living in damp wood: a study of five colonies

	$\bar{p}$ (CH_4)* (μg per termite per day)	n †	No. of colonies	$\bar{p}$ [$\mu\text{g CH}_4$ / (mg per termite per day)]
Workers	0.9 ± 0.1	32	5	0.5
Alates	0.09 ± 0.02	6	1	—
Control 1 (wet wood) ($\mu\text{g day}^{-1}$)	-0.06 ± 0.08	6	—	—
Control 2 (empty jar) ($\mu\text{g day}^{-1}$)	—	38	—	—
Zimmerman <i>et al.</i> (1982)	0.4 ± 0.1	87	2	0.2

Global production ($10^{12} \text{ g yr}^{-1}$)

$\bar{p}$ (CH_4) (μg per termite per day $^{-1}$)	Production ($10^{12} \text{ g yr}^{-1}$)	% Of total global production§	
0.9	65	12	This work
0.4	30	5	Zimmerman <i>et al.</i>
1.7	150	25	Ref. 7.

* The actual observed production of CH_4 in a jar was 25 times this value. There were 25 termites in the colony. $\pm$ values reflect 90% confidence limits of the mean representing the variation observed in the laboratory study.

† Number of CH_4 samples collected and analysed (June–September 1982 for our study).

‡ The production is based on a world population of 2×10^{17} termites. § The global production of CH_4 from all sources is about $550 \times 10^{12} \text{ g yr}^{-1}$.

¶ If there are 2.4×10^{17} termites in the world, and they produce $150 \times 10^{12} \text{ g yr}^{-1}$ of CH_4 , each would have to produce $1.7 \mu\text{g day}^{-1}$ of CH_4 , or 2–4 times more than laboratory studies.

are a lot of termites, and therefore may not reflect the world average for similar environments.

An alternative method to determine global production of CH_4 by termites was adopted by Zimmerman *et al.*⁸ as given by the following formula:

$$P_G = \delta \sum_{i=1}^N \epsilon_i m_{bi} A_i \quad (3)$$

where m_{bi} = the total biomass produced ($\text{g yr}^{-1} \text{m}^{-2}$) in the i th ecological region; A_i = global area (m^2) of the i th ecological region; ϵ_i = fraction of the biomass consumed by termites; and δ is the factor relating to the CH_4 produced to the biomass consumed and is obtained from the laboratory measurements of CH_4 emissions from termites. Twelve ecological regions were considered⁸ to arrive at $P_G = 150 \times 10^{12} \text{ yr}^{-1}$ and $N_\infty = 2.4 \times 10^{17}$. According to equation (2), however, this yields a $\bar{p} = 1.7 \mu\text{g}$ per termite per day or several times higher than the emission rates observed in the laboratory. Since ϵ_i and m_{bi} are extremely uncertain for each of the 12 regions, this method results in even greater uncertainties for the calculation of P_G , as evidence for example by the large and inconsistent final result of $P_G = 150 \times 10^{12} \text{ yr}^{-1}$ (refs 1, 8).

This inconsistency may be resolved if termites produce (about 3.5 times) more methane in natural conditions than in the laboratory; however, convincing data for such a phenomenon do not yet exist. Thus, the global production of CH_4 by termites is composed of the product of two quantities: $\bar{p}$, the average methane production per termite per yr, and N_∞ , the world population of termites: $\bar{p}$ is uncertain because termite production varies between species as well as within species. The between-species variation may depend on the difference of average weights, making a unit such as $\mu\text{g CH}_4$ per mg termite per yr a more suitable one for comparing methane production among different species and studies. $\bar{p}$ may also vary within species, depending on ambient temperature, humidity, and other environmental factors; thus methane production may well depend on season. Laboratory measurements made thus far do not take such variations into account. The large variabilities inherent in estimating the world termite population (N_∞) have already been discussed.

In view of these uncertainties, we constructed Fig. 1 to demonstrate the possible range of estimates for the global production of CH_4 by termites, consistent with laboratory measurements. Calculations of P_G ($10^{12} \text{ g yr}^{-1}$) based on the maximum and minimum emission rates (range) observed in the laboratory studies are shown as vertical lines and reflect an estimate of errors in $\bar{p}$ of equation (2). The more termites there are in the world, the greater the effect of errors in $\bar{p}$ is on estimates of global methane production. The solid line is based on $\bar{p} = 0.9 \mu\text{g}$ per termite per day (this work) and the dashed line is for $\bar{p} = 0.4 \mu\text{g}$ per termite per day (ref. 8); an estimate of P_G based on equation (3) is shown^{1,8}. This production rate, calculated by indirect methods using equation (3), is inconsistent with current laboratory data (see Table 1). The uncertainties in our present knowledge of $\bar{p}$, the production of CH_4 per termite per unit time, and N_∞ , the world termite population, allow a considerable range for the calculated global production of CH_4 by termites consistent with the two laboratory studies discussed here. From Fig. 1 it seems, however, that the methane production by termites is probably not more than 15% of the global yearly emissions. At that rate termites may be important in the global cycle of methane. At present global mass balance studies suggest that termites are likely to have a minor role in the global methane cycle, but more work is needed to prove this contention.

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Photosynthesis of picoplankton in the oligotrophic ocean

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Against a background of controversy concerning the absolute magnitude of biological productivity in the oligotrophic, tropical ocean^{1–4}, evidence is accumulating for the existence there of a population of minute, unicellular organisms collectively known as picoplankton^{5,6}. In the tropical Pacific, it has been estimated recently⁷ that cells passing a $1\text{-}\mu\text{m}$ screen (picoplankton) make a very substantial contribution to the rate of turnover of phytoplankton biomass. On the other hand, previous work in the tropical Atlantic⁸ led to the conclusion that particles in the size class $\leq 3 \mu\text{m}$ are fragments of larger cells and metabolically inert. We present here the first data on the photosynthetic characteristics of picoplankton collected at sea. This new evidence from the tropical North Atlantic supports the argument that the picoplankton contains a significant, metabolically-active, autotrophic component, capable of supplying about 60% of the total primary production in an open-ocean ecosystem.

Observations were made during July 1982 at 11 stations in the vicinity of the Mid-Atlantic Ridge west of the Azores, using methods described elsewhere^{7,9}. Samples were taken with a submersible pump from the chlorophyll maximum, the depth of which (65–89 m) was located by *in situ* fluorometry. Examination by auto-epifluorescence microscopy of the fraction passing a $1\text{-}\mu\text{m}$ screen and collected on a $0.4\text{-}\mu\text{m}$ Nucleopore filter revealed the presence of microbial cells in concentrations from 4.4×10^6 to 1.7×10^7 cells l^{-1} . In the scanning electron microscope (Fig. 1) the cells were seen to be of the coccoid type, possibly cyanobacteria of the genus *Synechococcus*^{5,6}. They were isolated and brought into culture using a standard medium for autotrophic algae¹⁰ (Woods Hole medium f) at 22°C and 14 W m^{-2} continuous light.

We compared the two size-fractions, separated by the $1\text{-}\mu\text{m}$ screen, according to the relationship between photosynthesis and light^{11–13}. Each curve is based on about 40 determinations

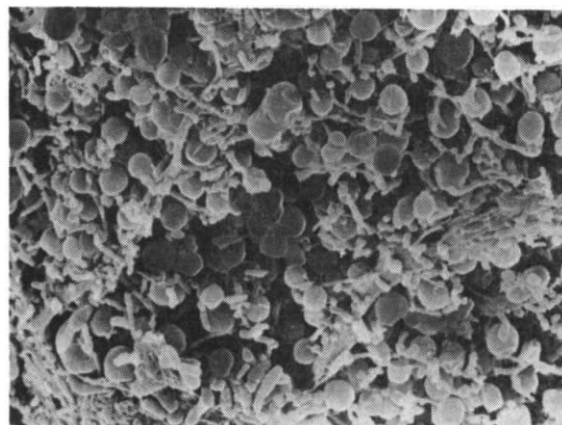


Fig. 1 Scanning electron micrograph of natural phytoplankton sample showing coccoid cells. Scale bar, $10 \mu\text{m}$.

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Table 1 Photosynthesis parameters for natural plankton assemblages from the chlorophyll maximum on Mid-Atlantic Ridge around latitude 36° N

Station	α (P^B/I)		P_m^B (mg C (mg Chl a) ⁻¹ h ⁻¹)		I_k (W m ⁻²)		I_m (W m ⁻²)		I_b (W m ⁻²)		B (mg ⁻³)	
	<1 μ m	>1 μ m	<1 μ m	>1 μ m	<1 μ m	>1 μ m	<1 μ m	>1 μ m	<1 μ m	>1 μ m	<1 μ m	>1 μ m
8	0.059	0.015	0.72	0.27	12	19	35	57	67	150	0.18	0.24
9	0.025	0.014	0.25	0.24	9.9	16	35	53	180	190	0.21	0.26
10	0.057	0.036	0.78	0.50	14	14	43	46	120	190	0.18	0.19
11	0.072	0.030	0.50	0.37	6.9	13	23	43	100	190	0.21	0.22
12	0.084	0.049	0.70	0.92	8.3	19	30	64	170	280	0.25	0.41
13	0.078	0.059	0.67	0.62	8.7	11	30	37	130	190	0.23	0.26
14	0.077	0.032	0.77	0.41	10	13	32	43	100	200	0.14	0.24
15	0.079	0.039	0.75	0.63	9.5	16	34	55	180	250	0.32	0.38
17	0.088	0.040	1.0	0.56	12	14	39	47	140	200	0.26	0.22
18	0.10	0.040	0.76	0.38	7.6	9.5	26	39	120	360	0.27	0.39
19	0.10	0.068	0.57	0.48	5.8	7.1	19	27	81	190	0.23	0.28
Mean	0.074	0.038	0.68	0.49	9.5	14	31	46	130	220	0.22	0.28

α is the initial slope; P_m^B is the assimilation number; I_k , I_m and I_b are irradiances and B is the chlorophyll biomass. All samples were separated by differential screening after incubation.

of photosynthetic rate measured along a light gradient (Fig. 2). Parameters describing the curves were found by nonlinear fitting^{14,15} to the equation of Platt *et al.*⁹. The parameter α , the slope of the curve at low light, is related to the efficiency of photosynthesis; P_m^B , the chlorophyll-specific photosynthesis at light-saturation (assimilation number) characterizes the photosynthetic capacity; I_m is the irradiance at which photosynthesis is optimal; the derived parameter I_k ($\equiv P_m^B/\alpha$) has been used as an index of photoadaptation in phytoplankton¹⁶; I_b is an index of susceptibility to photoinhibition.

For measurements of photosynthetic rate by the ¹⁴C method, we made the size-fractionation after the 2 h incubation. Pilot experiments showed that the photosynthetic capacity of the fraction passing a 1- μ m screen is reduced by about half for samples fractionated before incubation, perhaps because of mechanical damage to the cells. We verified the validity of the fractionated photosynthetic rate data by calculating for each experiment the quantity

$$\frac{\{P^B(<1 \mu\text{m}) \cdot B(<1 \mu\text{m}) + P^B(>1 \mu\text{m}) \cdot B(>1 \mu\text{m})\}}{\{B(<1 \mu\text{m}) + B(>1 \mu\text{m})\}}$$

as a function of available light and fitting to the same light-saturation equation. Here, B is the chlorophyll biomass and P^B is the chlorophyll-specific photosynthesis. The parameters so determined were compared with those obtained from experiments on unfractionated samples. The average ratio of the two sets of parameters was not significantly different from unity (mean 1.19; s.e. 0.14).

The photosynthesis parameters for the <1 μ m and >1 μ m fractions were quite different (Table 1). Assimilation number (P_m^B) and initial slope (α) were higher for the picoplankton fraction than for the larger fraction: the adaptation parameter (I_k), the optimal irradiance (I_m) and the photoinhibition parameter (I_b) were lower.

The results indicate that these autotrophic picoplankton are adapted to photosynthesize at a lower irradiance than is typical for the rest of the plankton community, a finding consistent with laboratory results for cyanobacteria¹⁷ and with the picoplankton data for the Pacific⁷.

The elevated rate parameters (P_m^B and α) for picoplankton are consistent with the general tendency for rate variables to increase with decreasing cell size¹⁸. Elevated α for picoplankton could also be a consequence of more efficient absorption of light at smaller cell size¹⁹. The size of these cells is similar to the wavelength of the available light, and could therefore confer a selective advantage. At light-saturation, when P_m^B is the relevant parameter, the implication of these results is that the picoplankton fraction (some 44% of the total chlorophyll biomass) contributes $\approx 53\%$ to the total primary production. On the other hand, because the cells were sampled from the chlorophyll maximum, where the ambient light level is below

saturation level, the parameter α is more important for calculation of the relative primary production *in situ*. Using insolation records made on the ship and an optical attenuation coefficient measured by underwater radiometry we estimate the average light available at the chlorophyll maximum during an 11 h light day as $\sim 6 \text{ W m}^{-2}$; we calculate that in these *in situ* conditions picoplankton contributes $\sim 60\%$ to the total primary production. The small difference between this value and that of 53% calculated for saturating light is one indication of the low irradiance at which picoplankton photosynthesis is optimized.

Using the fractionated particulate carbon data for the same samples we can convert the photosynthetic rates into carbon-specific growth rates, with dimensions [time⁻¹]. We find 0.15 day⁻¹ for the <1 μ m fraction and 0.05 day⁻¹ for the >1 μ m fraction. These numbers should be considered as tentative given the difficulty of extrapolating hourly production rates to daily rates and that of estimating the fraction of a particulate carbon sample that represents the autotrophic component. On the other hand, for the <1 μ m fraction, we derived a growth rate of the same order, but faster, when cell carbon was estimated directly from cell numbers (imprecise conversion factors are

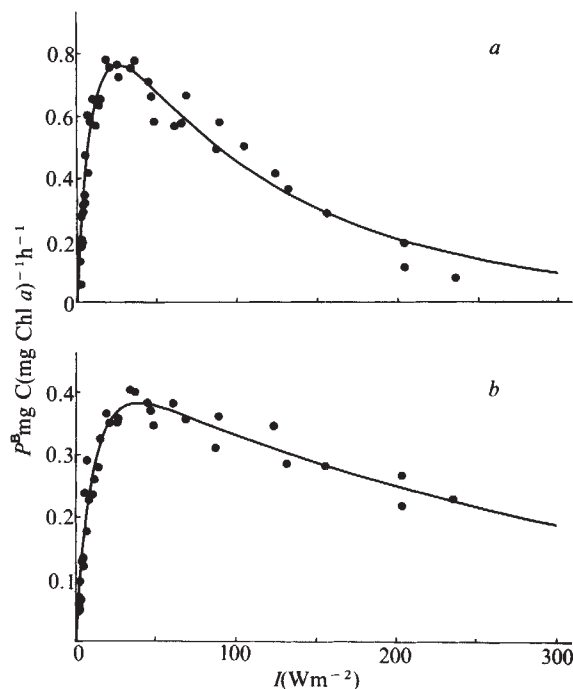


Fig. 2 Photosynthesis-light curves for two size-fractions of the same sample from station 18. *a*, <1 μ m fraction (sample 8771); *b*, >1 μ m fraction (sample 8770). Note that the ordinate scales differ.

involved in this calculation): we conclude that the results are probably accurate enough to support the case for relatively slow turnover in the oligotrophic ocean¹ rather than relatively fast turnover²⁰.

That cells from the picoplankton could be isolated and brought into culture extends the evidence presented by Li *et al.*⁷ against the case that the picoplankton fraction is either metabolically inert or is growing heterotrophically at the expense of ¹⁴C-labelled organic substrates excreted by larger phytoplankton. Our detailed documentation of the photosynthesis characteristics removes any doubt that there is an important autotrophic component to the picoplankton. The finding that photosynthesis of picoplankton saturates at irradiances close to those found at the chlorophyll maximum makes more credible the argument that the maximum is a consequence of growth *in situ* (see ref. 21). Taken together our results underline

the metabolic importance of the smallest organisms in the biomass spectrum^{3,22,23}. Although it has been traditional to suppose that pelagic organisms smaller than 1 µm lived a heterotrophic existence, we cannot now escape the conclusion that in these size classes, autotrophic growth also has an important role. To this extent we have to modify the way in which we perceive the structure and function of the pelagic ecosystem in the oligotrophic ocean. Finally, if the photosynthetic component of the picoplankton turns out to consist of only one or a few species of cyanobacteria (blue-green algae), conventional ideas²⁴⁻²⁶ on the relationship between species diversity, biomass turnover and ecosystem stability will require re-examination.

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Lectin-like polypeptides of *P. falciparum* bind to red cell sialoglycoproteins

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Attempts to control human malaria by immunological means could be compromised by antigenic variability within and between different strains of malarial parasites¹. A useful alternative approach might be to block parasite antigens which are important in the mechanisms of invasion of red cells. As the major human parasite *Plasmodium falciparum* is highly specific for human red cells, isolation of the proteins involved in the recognition of red cells by this parasite might be of particular value. Recent studies suggest that the major red cell sialoglycoproteins (SGPs), glycophorins A, B and possibly C, may carry the sites recognized by the parasite²⁻⁴. Furthermore, because certain carbohydrates present on SGPs such as *N*-acetylglucosamine are able to block invasion by the parasite⁵, they may be involved in the initial interaction between parasite and red cell. We have now identified parasite proteins which bind to SGP or *N*-acetylglucosamine on Sepharose 4B columns. Three proteins, of molecular weights (MWs) 140,000 (140K), 70K and 35K, seem to be specifically bound by *N*-acetylglucosamine.

Figure 1 shows the results of an experiment in which metabolically labelled proteins from late stage *P. falciparum* parasites were incubated with SGP coupled to Sepharose 4B beads and eluted with salt at a high concentration, with and without *N*-acetylglucosamine. Four major bands and several minor bands seemed to bind specifically to the column (Fig. 1, track 4). These bands include a 210K protein which has been associated with the surface coat of the merozoite⁶, two bands of

~140K and a lower MW protein at 35K. Stringent washing with a buffer containing a high concentration of salt and detergent removed the high MW band at 210K and the higher of the two bands at 140K (Fig. 1, tracks 2, 3). When *N*-acetylglucosamine was added to the washed column (Fig. 1, track 5) the lower band of the 140K doublet and a band at 35K were eluted (track 6). When the *N*-acetylglucosamine was added to the washing buffer, the *N*-acetylglucosamine eluate (track 1) also included the high MW band and the top band of the 140K doublet. However, only the lower 140K band and a band at ~35K are sugar specific. A control column was treated in the same manner, but without the sugar elution step; the two sugar-specific bands (140K and 35K) remained bound (as in track 5). Furthermore, these bands were not seen in the *N*-acetylglucosamine eluted column (as in track 6). This sample was balanced for counts with the other samples and this accounts for the enhanced appearance of minor components which were still bound to the column.

Figure 2 shows that from the initial total protein preparation (track 1), three major metabolically labelled *P. falciparum* proteins bind to *N*-acetylglucosamine-coupled Sepharose 4B beads (track 2). Washing the column with buffer containing a high concentration of salt did not remove any of these bands (track 3). Addition of *N*-acetylglucosamine to the washing buffer removed most of the three major bands (track 4); the major band at 140K was no longer present, but trace amounts of the other two bands at 70K and 35K remained. The *N*-acetylglucosamine eluate contained all three bands (track 5), although much of the protein was lost during dialysis. The three bands seem to be related to each other, possibly as a monomer, dimer and tetramer, because rerunning of the 140K band on a second SDS gel, also in reducing conditions, produced the same three bands at 140K, 70K and 35K. The three bands do not seem to be disulphide linked; an excess of 2-mercaptoethanol only led to partial dissociation of the 140K band into the smaller subunits. It is possible that these three proteins are part of a single polypeptide which has been cleaved by proteolysis or by the process of solubilization before the application of SDS-polyacrylamide gel electrophoresis. Alternatively, the 70K and 35K polypeptides could be processed products of the 140K polypeptide, in which case the sugar binding epitope is

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Fig. 1 The binding of metabolically labelled *P. falciparum* protein to SGP-coupled Sepharose 4B columns. A late-stage parasite protein preparation was made from a synchronized culture concentrated to 80–90% schizonts using a 3% gelatin solution (Plasmagel)⁹. The parasites were incubated with 200 Ci of ³⁵S-methionine (Amersham) in 5 ml (1,250 Ci mol⁻¹) methionine-free minimal essential medium (MEM) and 10% human serum for 3–4 h at 37°C (ref. 10). The culture was then washed three times in RPMI and solubilized in 2 ml lysis buffer (phosphate-buffered saline (PBS) pH 7.4, 0.25% Triton X-100, 5 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM TPCK + pepstatin + 20 g ml⁻¹ DNase I) at 37°C for 30 min and centrifuged at 100,000g for 45 min at 4°C. A 200-μl aliquot of the lysate (1 × 10⁵ c.p.m. μl⁻¹) was added to 400–600 μl of packed SGP-coupled Sepharose 4B beads. These were prepared by adding total red cell sialoglycoprotein¹¹ at 1 mg ml⁻¹ to 1 ml activated Sepharose 4B beads (Pharmacia) and equilibrating with PBS, pH 7.4. The columns were incubated with the *P. falciparum* lysate for 30 min at 37°C then at 4°C overnight. Four major bands of parasite proteins were bound (Col. start, track 4) as well as several minor bands. Stringent washing with 0.5–1.0 M NaCl and 0.25% Triton X-100 in PBS (Dulbeccos A and B Oxoid) removed the high MW protein at 210K and the higher MW protein at 140K (Col. washed, track 5), leaving two major bands; the lower of the 140K doublet and 35K protein. The eluate (Washes 1, 2; tracks 2, 3) contained the two high MW proteins removed from the starting column. When 0.3 M *N*-acetylglucosamine was added to the washed column the remaining two major proteins are removed (Col. final, track 6). When *N*-acetylglucosamine was added to the washing buffer, the eluate includes all four major proteins (*N*-AcGlu eluate, track 1). Consequently, only the lower 140K protein and a protein at ~35K are sugar specific. All washes and sugar eluates were dialysed against PBS and concentrated in a minicon concentrator (Amicon). All samples were boiled for 2 min in sample buffer (50 mM Tris HCl; 5% 2-mercaptoethanol, 1% SDS, 10% glycerol, 2 mM PMSF, 1 mM EDTA), applied to SDS-polyacrylamide gel electrophoresis, run at 40 mA and fluorographed. ¹⁴C-labelled standard molecular weight markers were included on each gel.

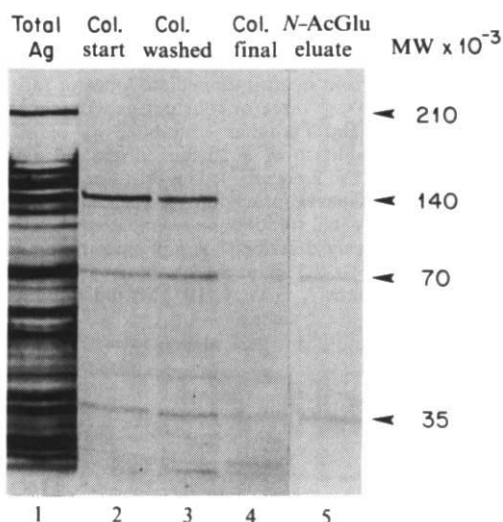
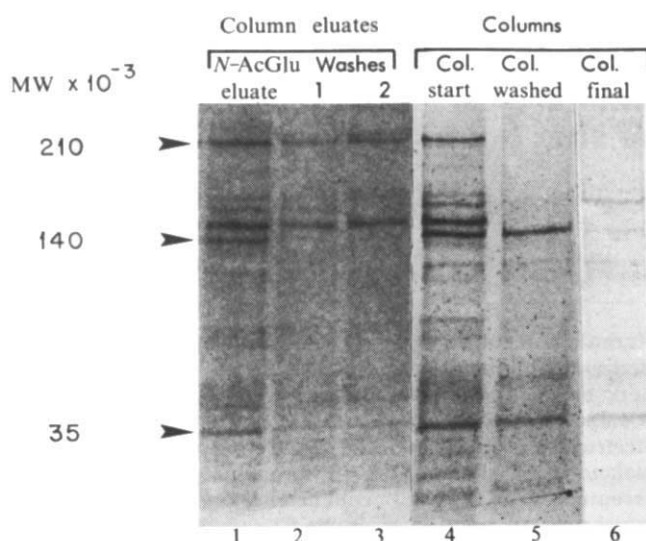


Fig. 2 The binding of metabolically labelled *P. falciparum* proteins to *N*-acetylglucosamine-coupled beads. Track 1 shows the unfractionated proteins. Only three of these proteins (MW 140K, 70K and 35K) bind to *N*-acetylglucosamine-coupled beads (Col. start, track 2). Washing buffer failed to remove them (Col. washed, track 3) but *N*-acetylglucosamine (0.3 M) eluted all three (Col. final, track 4). The 140K, 70K and 35K proteins were present in the *N*-acetylglucosamine eluate (track 5). Samples from test and control columns were prepared as described for SGP columns. *N*-acetylglucosamine- and lactose-coupled beads were obtained from Pierce. No binding was seen to lactose-coupled or to non-coupled Sepharose control columns (not shown). Track 1 shows metabolically labelled proteins prepared from *P. falciparum* schizont. Only three of these proteins (MW 140K, 70K and 35K) bind to *N*-acetylglucosamine-coupled beads (Col. start, track 2). Washing buffer failed to remove them (Col. washed, track 3) but added *N*-acetylglucosamine (0.3 M) eluted all three of these proteins (Col. final, track 4). The 140K, 70K and 35K proteins were present in the *N*-acetylglucosamine eluate (*N*-AcGlu eluate, track 5). Samples from test and control columns were prepared as described for SGP columns. No binding was seen to lactose or to non-coupled Sepharose control columns (not shown).

present on all three proteins. Lactose-coupled beads and uncoupled Sepharose beads served as controls and did not bind any protein which could not be removed with extensive washing in buffer containing a high concentration of salt.

Our studies indicate that there are at least four polypeptides of *P. falciparum* schizonts which bind to glycophorin, two of which specifically bind to *N*-acetylglucosamine coupled to Sepharose beads. Other recent studies indicate that the glycophorins and their associated sugars may be of considerable importance in the interaction of *P. falciparum* with the surface of the human red cell membrane^{4,7}. It has been suggested that invasion may follow a two-stage process in which the parasite binds with sugar molecules by a lectin-like interaction and that this leads to its correct orientation with a more specific binding site on one or more of the glycophorin molecules^{4,7}. The identity of the oligosaccharides and/or adjacent molecules which are bound by the protein or proteins that we have isolated may reveal which portion of the glycophorin molecules makes up the definitive merozoite attachment site. It is possible that the proteins which bound to SGP on the column represent the components of a multistep process. For example, the two higher MW proteins may be involved in a reversible association of the merozoite to the red cell, whereas the sugar-specific protein(s) may bind with a higher avidity and provide a more stable attachment. Because of the specificity of the interaction between red cell and parasite it will be particularly interesting to examine the effects of antibodies prepared against these antigens on the invasion of human red cells by *P. falciparum*. Interestingly, Perrin and Dayal⁸ have described an inhibitory monoclonal antibody which binds a 140K *P. falciparum* protein.

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GABA acts directly on cells of pituitary pars intermedia to alter hormone output

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Recent immunohistochemical evidence from the rat, indicating that γ -aminobutyric acid (GABA)-containing fibres of central nervous origin project to the pars intermedia of the pituitary^{1,2}, prompts inquiry into the function of this innervation. There is electrophysiological evidence that GABA acts directly on melanotrophs isolated from rat, through bicuculline-blockable receptors, to increase Cl^- conductance and thereby drive the membrane potential towards the Cl^- equilibrium potential in these cells, resulting in depolarization at rest or reduction of the depolarization caused by excess K^+ (ref. 3). As voltage-dependent Ca channels can participate in the regulation of secretion in these cells⁴, we have now examined the effect of GABA on hormone output and find that it first stimulates and then inhibits spontaneous secretion of melanocyte-stimulating hormone (MSH) and inhibits K^+ -evoked secretion. Moreover, our pharmacological evidence suggests that similar receptors are involved in the secretory and the electrophysiological responses. A function of the GABAergic innervation may therefore be to regulate hormone output by acting directly on the melanotrophs, and this regulation may be affected by the changes in electrical properties induced by GABA.

Because the relevant morphological^{1,2} and electrophysiological³ evidence has been obtained from the rat, we have used rats for most of our experiments. Pars intermedia cells from rat neurointermediate lobes were dispersed, maintained *in vitro* in short-term culture, and packed in a column that allowed continuous perfusion and measurement of hormone output. Previous experiments of this sort have shown that the cells exhibit basal secretory activity that is largely Ca^{2+} dependent⁴, and that secretion can be stimulated, again in a Ca^{2+} -dependent manner⁴, by excess K^+ (refs 4, 5), which depolarizes the cells⁶. This stimulation of secretion is considered⁴ to be the consequence of Ca^{2+} entry through the voltage-dependent Ca channels demonstrated electrophysiologically⁷. These factors and the demonstration that the Ca ionophore A23187 stimulates secretion⁸ suggest that the endocrine cells of the pars intermedia belong to the large subclass of secretory cells that possess voltage-dependent Ca^{2+} entry mechanisms which are important in stimulus-secretion coupling⁹.

When tested on such isolated pars intermedia cells secreting spontaneously, GABA (10^{-4}M) on each occasion stimulated secretion briefly and then depressed it (Fig. 1a). In five trials on four batches of cells, the peak secretory rate, measured as MSH activity, rose during exposure to GABA to $300 \pm 44\%$ (mean $\pm$ s.e.m.) of the basal secretory rate during the 5-min period before GABA addition. The inhibitory effect of GABA that followed stimulation (Fig. 1a, c) in these five trials was assessed by comparing hormone output during the 10th minute of exposure to GABA with the mean of the basal outputs immediately before GABA and 10–15 min after removing GABA when the baseline had stabilized. Such measurements showed that GABA reduced output to $38.8 \pm 5.2\%$ of the basal rate. These responses to GABA were not apparent when the perfusion solution lacked Ca^{2+} (Fig. 1b, c). Two additional trials in another batch of cells yielded similar results.

When tested on cells stimulated to secrete by excess K^+ (50 mM), GABA (10^{-4}M) had only an inhibitory effect (Fig. 2) which was evident within 2 min, that is, at a time when the effect of GABA alone was to stimulate secretion (compare with Fig. 1). In six such trials on three batches of cells, peak output

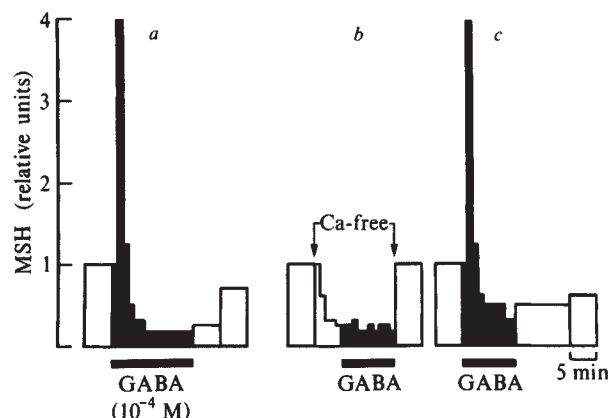


Fig. 1 Biphasic effect of GABA on basal secretion from cells isolated from rat pars intermedia and its dependence on Ca^{2+} . *a*, Typical response to GABA (10^{-4}M) which initially stimulates and then inhibits secretion. *b*, *c*, Another preparation showing the dependence of the GABA effect on Ca^{2+} . *b*, Lack of effect of GABA (10^{-4}M) during perfusion with Ca^{2+} -free medium. *c*, Subsequent control response 30 min after reintroducing Ca^{2+} . Records show output of MSH biological activity from continuously perfused columns of dispersed cells. Perfusates for assay were collected for 1-min periods during exposure to GABA (horizontal bars) or Ca^{2+} -free solution (arrows), otherwise for 5- or 10-min periods (calibration bar). Tests were made only when successive 5-min collection periods showed that basal secretion had attained a steady level. Control experiments have shown that at such times, measurements of hormone output at 1 or 5 min intervals are indistinguishable. On the vertical axis, 1 equals 200 pg ml^{-1} standard α -MSH in *a*, and 120 pg ml^{-1} in *b* and *c*. Pars intermedia cells were dispersed from neurointermediate lobes of rats (male Sprague-Dawley, ~300 g, three or four per experiment) by the method of Hopkins and Farquhar¹⁶ modified by omission of neuraminidase and addition of a 15-min incubation with collagenase (Sigma type IV; 2 mg ml^{-1}). The cells were cultured for 1–9 days before use. General procedures for cell culture, column preparation and perfusion, solution preparation, and sample bioassay were as previously described⁴ except that the amount of BioGel was reduced to 0.5 ml packed volume. Flow rate was maintained at 0.5 ml min^{-1} . GABA (10^{-4}M) did not affect the assay.

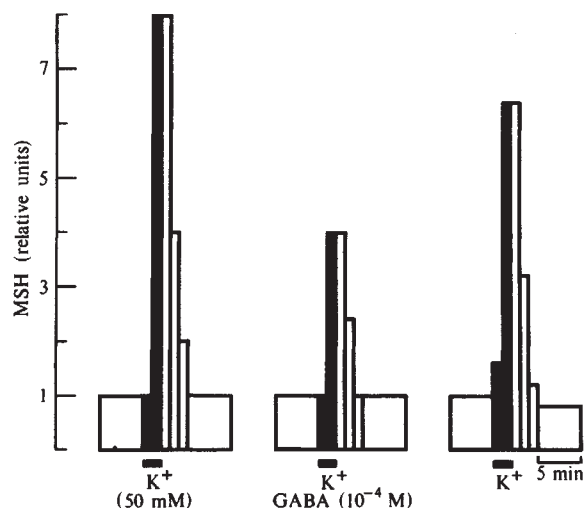


Fig. 2 Inhibitory effect of GABA on K^+ -stimulated secretion. Three successive secretory responses to 50 mM K^+ applied for 2-min (horizontal bars) at 35-min intervals. During the second response, GABA (10^{-4}M) was added together with the excess K^+ . Note that the GABA-induced inhibition of the secretory response to excess K^+ occurs at a time (min 2) when the effect of GABA alone is stimulant (compare with Fig. 1). On the vertical axis 1 equals 60 pg ml^{-1} standard α -MSH. Other procedures as in Fig. 1 legend.

of hormone in response to excess K^+ given with GABA was only $61.1 \pm 5.5\%$ of the subsequent control response to excess K^+ alone.

Muscimol, which acts as an agonist at GABA receptors linked to Cl channels¹⁰ of the sort found in melanotrophs³, mimicked the stimulant effect of GABA on spontaneous secretion in each of seven batches of cells, secretion rising to $304 \pm 40\%$ ($n = 5$) of control with 10^{-6} M muscimol and to 500 and 800% of control with 10^{-5} M muscimol. Furthermore, the stimulant effects of both GABA and muscimol were completely blocked by bicuculline (2×10^{-5} – 10^{-4} M: $n = 3$, $n = 2$, respectively), which acts as an antagonist at the same type of GABA receptor¹⁰ and which itself had no effect on basal secretion. In addition, muscimol (10^{-5} M) mimicked the inhibitory effect of GABA on K^+ -evoked secretion, reducing secretion to $60.5 \pm 11.8\%$ of the subsequent control response to excess K^+ ($n = 3$).

We have obtained similar results with GABA in experiments on cells isolated from the pituitary pars intermedia of mice (female, CD1 strain, 7–10 weeks old). The cells were isolated and perfused in the same way as were the rat cells (see Fig. 1 legend). GABA (10^{-5} – 10^{-4} M) caused a brief intense stimulation of secretion to $453 \pm 71\%$ ($n = 6$) of the basal level and inhibited the response to 50 mM K^+ , reducing it to $38.3 \pm 7.7\%$ ($n = 4$) of the subsequent control response to excess K^+ .

The present results provide the first demonstration that GABA affects melanotroph secretion. Although GABA has been stated to inhibit MSH output from intact pituitary glands or lobes of rat and frog but not of mouse^{11,12}, no evidence for these statements has been provided. Our results show that GABA acts directly on cells isolated from the pars intermedia of both rat and mouse and that its effects are complex and involve stimulant as well as inhibitory components. This might be explained by the several electrophysiological effects of GABA on these cells³. First, it seems likely that the stimulant effect of GABA on basal secretion is attributable to its depolarizing effect³. Thus, GABA's stimulant effect, like that of a depolarizing concentration of K^+ (ref. 4), is Ca^{2+} dependent. (Unpublished observations by P.S.T. and W.W.D. show that the absence of Ca^{2+} does not prevent GABA from depolarizing the pars intermedia cells.) Furthermore, this stimulant effect of GABA is blocked by bicuculline, which abolishes the increase in Cl^- conductance and the resulting GABA-induced depolarization³. The brevity of this phase of increased secretion in response to GABA is consistent with other evidence indicating that the voltage-dependent Ca channels in rat pars intermedia cells become rapidly inactivated⁴. Second, the phase of inhibition of spontaneous hormone output that follows the evanescent stimulant effect of GABA might be correlated with suppression of spontaneous electrical activity as reflected in the observed arrest of action potentials³. It cannot be due to depletion of releasable hormone, for no such inhibitory phase is evident on stimulation with excess K (compare Figs 1 and 2; see also ref. 4). Finally, the inhibitory effect of GABA on K^+ -evoked secretion may again reflect increased Cl^- conductance and the consequent reduction of the depolarization³ produced by excess K^+ . The observation that muscimol also inhibits K^+ -evoked secretion is consistent with this interpretation.

Regardless of whether the diverse electrophysiological effects fully explain the secretory responses, it is evident that GABA has direct actions on melanotrophs that cause rapid changes in hormone output. This, in conjunction with the morphological evidence of GABAergic innervation of the region^{1,2}, allows the further conclusion that the brain may use GABAergic mechanisms, in addition to others already postulated^{13–15}, to modify directly hormone output from these endocrine cells.

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Neuronal correlates of sleep, wakefulness and arousal in a diurnal insect

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The discovery that various states of sleep, rest, wakefulness and arousal in man can be correlated with specific forms of the electroencephalogram¹ has led to intensive studies of these states, mostly in mammals^{2–5}. Today it is generally accepted that circadian sleep–wakefulness cycles occur in mammals and birds^{2,3,6}. Behavioural observations on sleep in moths have also been published⁷; many other invertebrates demonstrate rest/activity cycles⁸. Circadian sensitivity fluctuations in both central⁹ and peripheral^{10–15} components of the visual system of various nocturnal arthropod species have been demonstrated. We now report that long-term, extracellular, single-unit recordings from optomotor interneurons in the optic lobes of forager honey bees reveal an oscillation in their sensitivity to moving visual stimuli^{16,17}. The oscillation displays properties typical of a circadian rhythm^{6,18}. The sensitivity of the neurones is higher during the subjective day than during the subjective night. The locomotor activity of individual, fixed walking forager bees shows a similar circadian oscillation and is also higher during the subjective day. Visual and mechanical stimuli can act directly on the interneurons and restore their sensitivity during times of reduced neuronal responsiveness. A comparison with results available for mammals makes it likely that the neuronal phenomena presented here are correlates of the bee's circadian sleep–wakefulness rhythm.

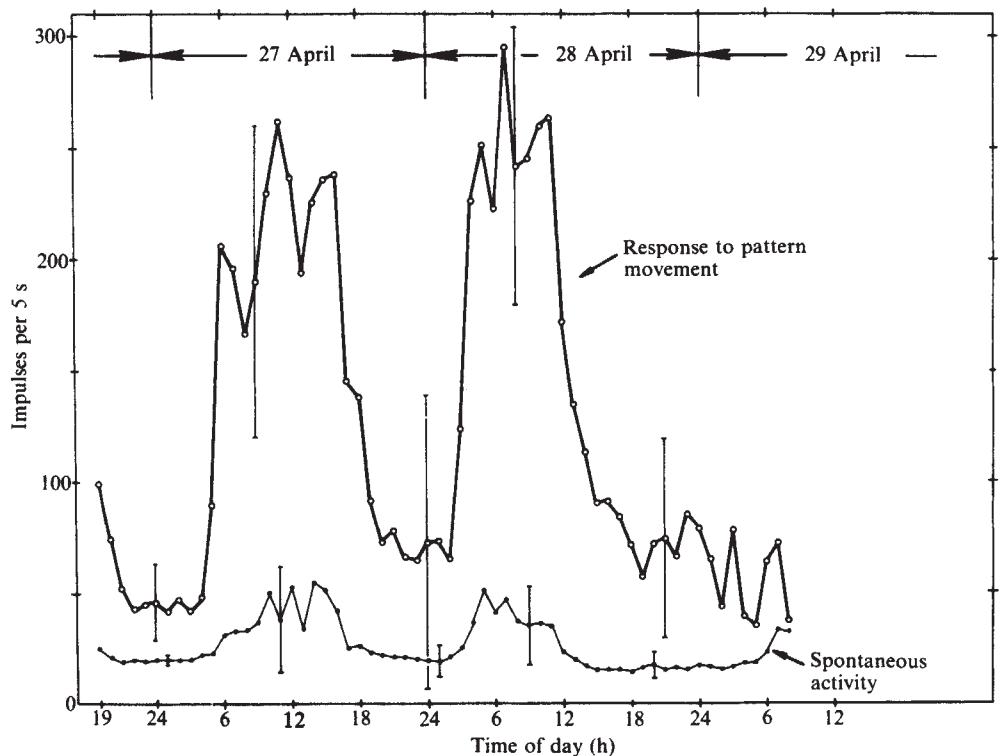
The neurones investigated here are wide-field visual interneurons located in the lobulae of forager honey bees (*Apis mellifera*). They are directionally selective for stimuli which move horizontally and belong to the bee's optomotor system. There are good reasons to believe that these neurones are, in fact, the previously identified HR-cells¹⁹.

Details of the experimental set-up, preparation and recording procedures have been reported previously^{19,20}. In the present study, extracellular, etched steel electrodes were used. Before electrode penetration, the bee received a large meal of diluted honey and the antennae were removed. The animals survived for a maximum of 4.5 days.

The sensitivity of the neurones to light of a given wavelength varies according to the time of day: the sigmoid response–log intensity function (which has a positive slope) shifts along the abscissa towards higher intensities in the evening. From Fig. 1 it is evident that this fluctuation in sensitivity actually represents an endogenous, circadian oscillation. The only source of light throughout the experiment was a vertical striped pattern. It was illuminated continuously with an intensity which elicited half of the maximum response on the day on which the experiment began. The bee which provided the data for Fig. 1 survived for 2.5 days.

The circadian changes in neuronal sensitivity are probably related to the sleep–wakefulness rhythm of forager bees. The

Fig. 1 Response of an optomotor interneurone of the bee to a standard stimulus (horizontal movement of a striped pattern of constant intensity), plotted against the time of day. The circular boundary of the pattern, which was viewed with the right eye only, subtended a visual angle of 20.8° . Contrast frequency, 3 Hz; pattern wavelength, 9.6° ; luminance at the eye, $17 \times 10^{-1} \text{ cd m}^{-2}$; colour temperature, $\sim 1,920 \text{ K}$. During the entire course of the experiment, the pattern was moved in the neurone's preferred direction for 5 s every minute and the spike activity recorded. The spontaneous activity was measured in the 5-s interval immediately before pattern movement. Each point on the graph represents the average of 60 individual values. The recording started in the evening. The sensitivity of the neurone obviously follows a circadian rhythm. Here the period length is $\sim 22 \text{ h}$, in other experiments it was $\sim 23 \text{ h}$. The vertical bars represent standard deviations. The bee died after the last value shown on the graph was obtained. On the day before the experiment sunrise occurred at 05:14 h, sunset at 19:34 h.



neurones show increased sensitivity during the subjective day, that is, when the motor activity of the animals is high. Figure 2 shows the locomotor activity of an individual, fixed forager bee walking on a treadwheel and maintained in continuous darkness. A free-running, circadian oscillation with maximum activity occurring during the subjective day is evident. A similar result was obtained with constant illumination, that is, in conditions comparable with those prevailing in the electrophysiological experiments. These results are in good agreement with measurements made on individual, unrestrained worker bees²¹.

The occurrence of short bouts of sleep during time-spans of motor activity in mice⁵ may be analogous to our discovery of occasional brief (5–15 min) episodes of reduced neuronal responsiveness during the day. These episodes are apparent in the raw data of Fig. 1. In the hive, forager bees often crawl into empty comb cells and rest²².

If the time-spans of reduced neuronal sensitivity for motion stimuli do, in fact, correspond to sleeping times, arousal stimuli should restore the neurones' sensitivity. Figure 3 shows that this expectation was confirmed. The bee which provided the data for Fig. 3 survived for 4.5 days. The record in Fig. 3a was made during the first subjective day, Fig. 3b–e during the first subjective night. These data make the existence of a general arousal system in the honey bee plausible. This system obviously acts directly on the optomotor interneurons themselves (Fig. 3c). An arousal system, functionally similar to the midbrain reticular activating system of mammals, has been postulated for the locust, *Schistocerca*²³.

The electrophysiological phenomena following arousal described above are remarkably similar to some found in cats²⁴. Extracellular recordings made from single visual neurones in the lateral geniculate body of sleep-deprived cats revealed an increase, on arousal from slow-wave sleep, in both the evoked responses to visual stimuli and the level of spontaneous activity of the neurones. In the striate cortex, there was also usually an increase in the evoked response following arousal, often accompanied by a decrease in spontaneous activity which resulted in an improved signal-to-noise ratio.

It is generally assumed that the circadian sleep–wakefulness rhythm of higher vertebrates is generated in the central nervous

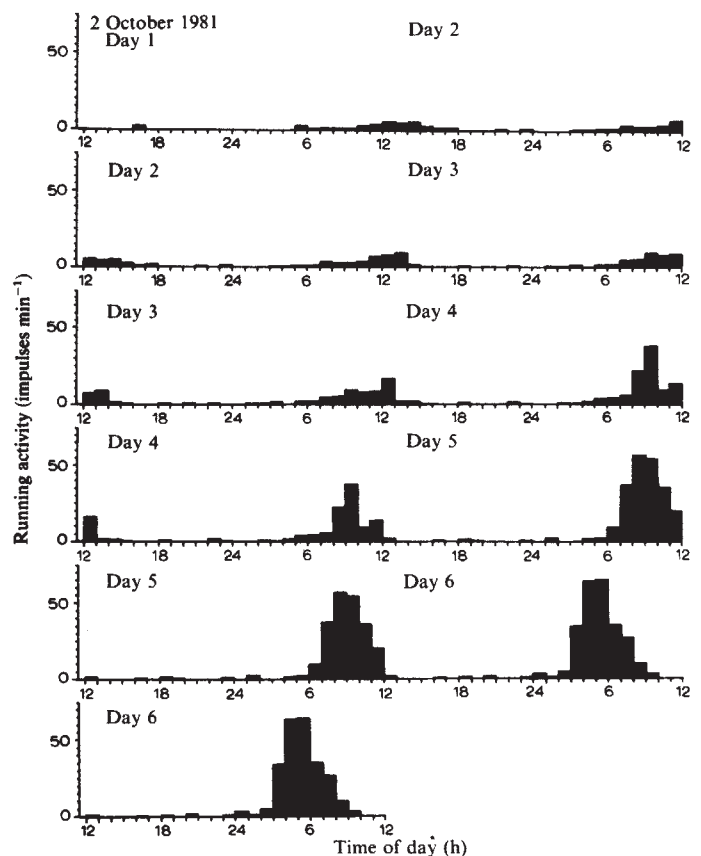
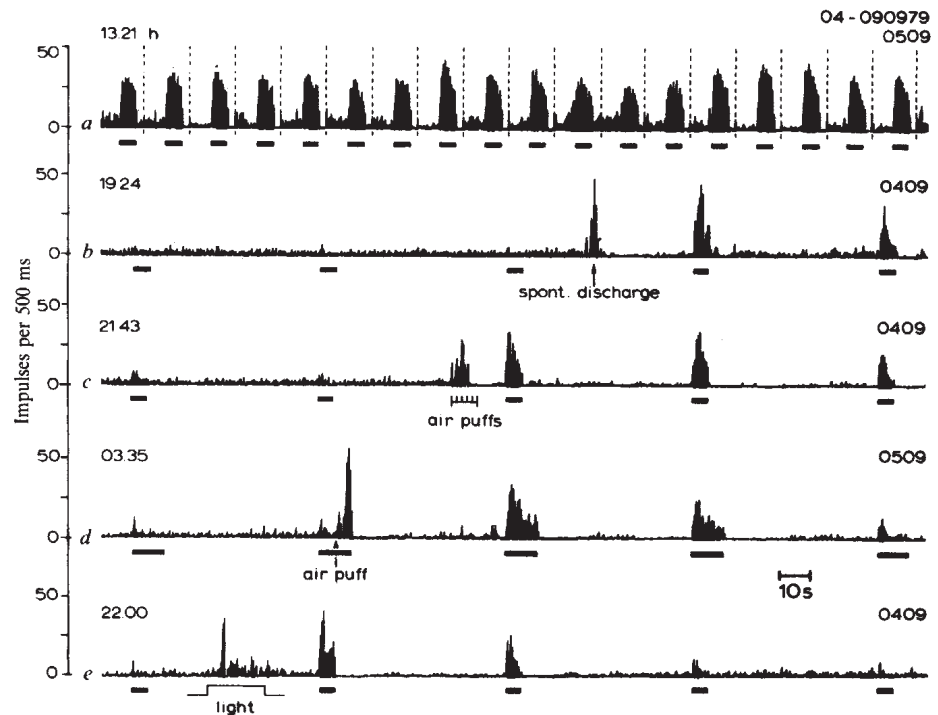


Fig. 2 Pattern of locomotor activity (walking speed) of a single, fixed forager bee, maintained in continuous darkness, as a function of the time of day. The bee was fixed at the thorax above a treadwheel of 2.5 cm diameter and could drink honey *ad libitum* from a glass capillary tube. The animal's head could move freely. Three impulses represent a distance of 7.85 cm. Sunrise on the day before the experiment occurred at 06:25 h, sunset at 18:06 h. In continuous darkness, the period of the free-running locomotor activity was $\sim 23.5 \text{ h}$. These data were collected by Hans Bösebeck.

Fig. 3 Excerpts from peristimulus time histograms which were recorded continuously from an optomotor interneurone. *a* Shows the reaction of the neurone to a series of 18 pattern movements (black bars) over 18 min. During the day, the neurone responded for several hours to pattern movement occurring at 1-min intervals. As the response of the neurone does not habituate, these optomotor interneurons are very different from the lobular giant movement detector/descending contralateral movement detector (LGMD/DCMD) system of the locust, *Schistocerca*, which characteristically shows rapid habituation²⁹⁻³¹. In the evening and during the night, the neurone responds weakly or not at all to the normally very effective pattern movement stimulus (*b-e*). Bursts of spontaneous discharge (*b*) often occur during such insensitive phases. Such spontaneous bursts are always followed by a strong response to pattern movement. The spontaneous bursts seem to be reflections of a process which acts directly on the neurones and which leads to an increase in their sensitivity to pattern motion. It is likely that these spontaneous discharges are triggered by proprioceptive (mechanoreceptive) input: puffs of air directed onto the mechanoreceptive sensory hairs on and around the left eye of the bee (the antennae had been removed before electrode penetration) cause an increase in the discharge rate of the optomotor interneurone (*c*). One could therefore describe these neurones as multimodal. Even a single air puff is sufficient (*d*): it produces a small burst and after a delay of less than 2 s there is a strong response to pattern movement. Both the relatively short latency of the increase in visual sensitivity and the fact that the neurone responds to the mechanical stimulus indicate that the effects due to mechanoreceptive stimulation act on the optomotor interneurons themselves. Furthermore, record *d* shows that the visual response declines after a relatively short time. This response reduction is probably not habituation—see record *a*—but rather a fading-away of the arousal effect caused by the mechanical stimulus. *e* Shows that a pulse of light (of physiological intensity) delivered to the eye (left eye) which does not see the pattern, and to the ocelli, also leads to an increase in the impulse activity. This is then followed by a transient increase in the sensitivity of the neurone. Bin width, 500 ms. Time scale for the abscissae is shown below trace *d*. Experimental conditions as in Fig. 1.



system (CNS)^{2,3}. We have compelling evidence that this is also the case in the bee. Recent long-term recordings of the bee's electroretinogram²⁵ have demonstrated a circadian sensitivity oscillation in the eye. Maximum sensitivity occurs during the subjective night (T. Labhart and A. Simmler, personal communication). Because this peripheral oscillation is in antiphase with the neuronal sensitivity rhythm, it cannot be the cause of the latter. Additional evidence is provided by our observation that, in continuous darkness, the resting neuronal discharge continues to show daily fluctuations whose sign and amplitude vary like those of the spontaneous activity during pattern illumination.

The only previously published reports describing circadian sensitivity fluctuations in afferent, higher-order visual interneurons of arthropods deal with the 'sustaining fibres' of decapod crustaceans^{9,26}. Maximal neuronal sensitivity in these nocturnal animals occurs during the subjective night and, in the crayfish *Procambarus*, is co-phasic with the circadian sensitivity oscillations in the eye^{9,10}. In both the diurnally active *Apis* and the nocturnally active *Procambarus*, peaks of maximal neuronal sensitivity coincide with the animal's natural activity times. The apparently paradoxical situation of the bee's electroretinogram rhythm might be explained as follows. During times of rest (sleep), animals can 'afford' to let parts of their sensory systems become largely insensitive, provided that they can be reactivated quickly. The bee's optomotor neurones represent such a system (Fig. 3*d*). In arthropod eyes, on the other hand, changes in sensitivity due to dark adaptation generally proceed slowly²⁷. Thus, it would be advantageous for both diurnal and nocturnal animals to raise their eyes' sensitivity at night. The slow part of the system would then be in an optimal condition to function on demand. (In *Heliconius*, a diurnal tropical butterfly, the eyes are also maximally sensitive at night²⁸.) Such an antiphasic relationship between the sensitiv-

ity of the eyes and of parts of the CNS may prove to be typical of diurnally active arthropods.

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Possible role of acetylcholinesterase in regulation of postsynaptic receptor efficacy at a central inhibitory synapse of *Aplysia*

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Most of the effects of acetylcholinesterase (AChE) on synaptic transmission are considered to be related to its acetylcholine (ACh) hydrolysing properties. This is clearly apparent from changes which occur in the characteristics of the miniature endplate potential and of the endplate potential at neuromuscular junctions when AChE is inhibited¹⁻⁴ and during the development of enzymatic AChE activity at maturing synapses⁵. However, we report here that after inhibiting AChE in a cholinergic synapse in *Aplysia*, we found an increase not only in postsynaptic responses to presynaptic stimulation and to ionophoretic application of ACh on postsynaptic receptors, but also to ionophoretic application of carbachol. This could not be explained by the inhibition of the ACh hydrolysing function of the enzyme, as carbachol is not hydrolysed by AChE. A possible explanation of these observations is that inhibition of the enzyme affects a property of the ACh receptor (AChR) itself.

Experiments were performed on isolated and desheathed ganglia of *Aplysia californica* (Pacific Bio Marine Company, California). We used two identified cholinergic interneurons which induce inhibitory Cl⁻ dependent postsynaptic responses (H-type response)⁶ in several identified postsynaptic cells in the buccal ganglion⁷⁻¹⁰, and R₁₅, which is situated in the abdominal ganglion and which has cholinergic receptors opening cationic channels (D-type response)⁶.

Current recordings of postsynaptic responses evoked by a presynaptic spike or ionophoretic application of the agonist were performed using a classical voltage clamp. Because in these central cells it is not possible to record individual postsynaptic currents resulting from the release of quanta from a given interneurone, quantal aspects of the transmission in the buccal ganglion were obtained by an indirect method as previously described^{11,12}. Briefly, both pre- and postsynaptic neurones were simultaneously voltage-clamped to holding potentials of -50 mV and -80 mV, respectively. When the voltage-dependent Na⁺ conductance was blocked by tetrodotoxin (TTX), a depolarization of the presynaptic neurone gave rise to a prolonged release of the transmitter from the terminal located a few hundreds of micrometres from the soma. The response recorded in the postsynaptic cell showed fluctuations or noise resulting from the summation of discrete events, representing miniature postsynaptic currents (m.p.s.c.s). Using a computer, it was then possible to apply to this response (l.d.i.p.s.c., long depolarization-induced postsynaptic current) statistical analysis and calculate from Campbell's theorem and the power density spectra¹³, respectively, the amplitude of a single miniature postsynaptic response and the time constant of its decay.

Similarly, open time of the Cl⁻ channel was calculated using a fast Fourier transform from the responses to prolonged ionophoretic applications of ACh or carbachol onto the somatic receptors^{12,14}. As in most *Aplysia* central neurones⁶, AChRs on the buccal postsynaptic cells are not confined to the synapse but are also distributed on the soma, which is devoid of synaptic contacts. ACh and carbachol were alternately applied at the same membrane spot using a constant current source and a

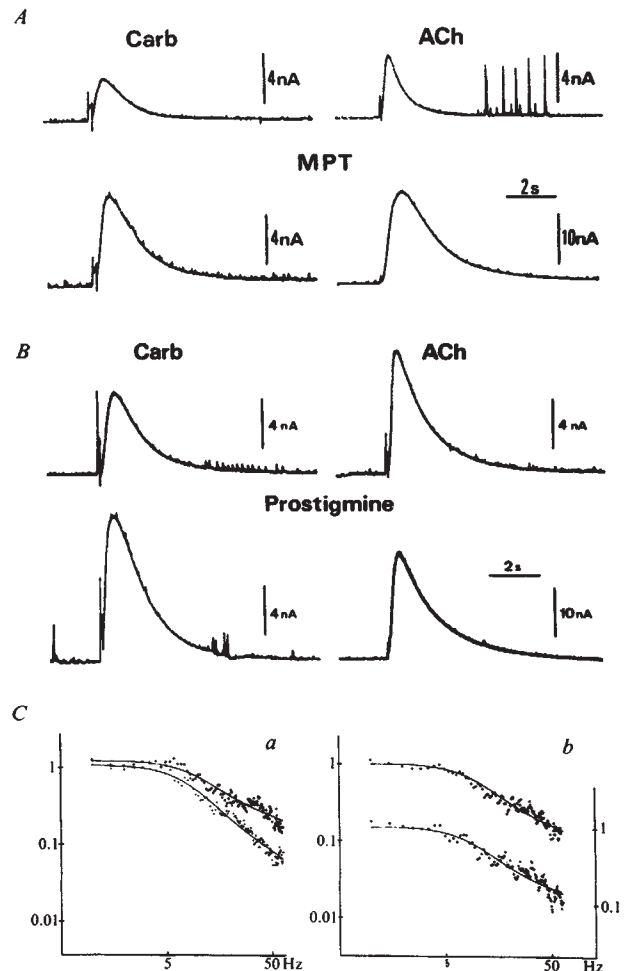


Fig. 1 Current responses to ionophoretic application of ACh (right column) and carbachol (left column) on the soma of the same postsynaptic cell (H cell) in the buccal ganglion. The cell was voltage clamped at -80 mV. **A**, MPT was bath-applied for 10 min and after washing for 1 h 45 min, the amplitude of both responses was increased (lower traces). **B**, In another preparation prostigmine was bath-applied for 10 min. Note the increase of amplitude of both responses (lower traces). **C**, **a**, Spectral density calculated from a fast Fourier transform of Cl⁻ channel noise: ionophoretic application on the same membrane spot of ACh (lower trace) and of carbachol (upper trace). Double Lorentzians (solid lines) were fitted to the data points. The open time of the slow component was 21 ms for ACh and 17 ms for carbachol (temperature, 20 °C). **b**, Spectral density of carbachol Cl⁻ channel noise before (upper trace) and after application of phospholine (2 × 10⁻⁴ M) and prolonged wash (lower trace). The two power spectra are superimposable. (Open time of the slow component: 16.8 ms for the test and 17.7 ms after treatment.) Power spectral amplitudes are shown normalized. In **b**, the left-hand scale is for one upper trace and the right-hand scale for the lower trace. For **a** and **b** 10 spectra were averaged from traces of 1,024 points at 2-ms intervals.

double-barrelled or two single electrodes filled with the agonists at 1 M concentration. Braking current was applied to the electrodes not in use. Before application of the drugs, the position of the ionophoretic electrodes was adjusted to obtain the maximal response for a given injecting current and extreme care was taken to ascertain that, in the absence of additional experimental manipulations, repeated injections gave uniform responses.

In all experiments, the Cl⁻ reversal potential of the postsynaptic cells was monitored frequently, as was the temperature. Current amplitudes were routinely transformed into conductances. The preparation was continuously perfused with normal saline or saline to which drugs were added.

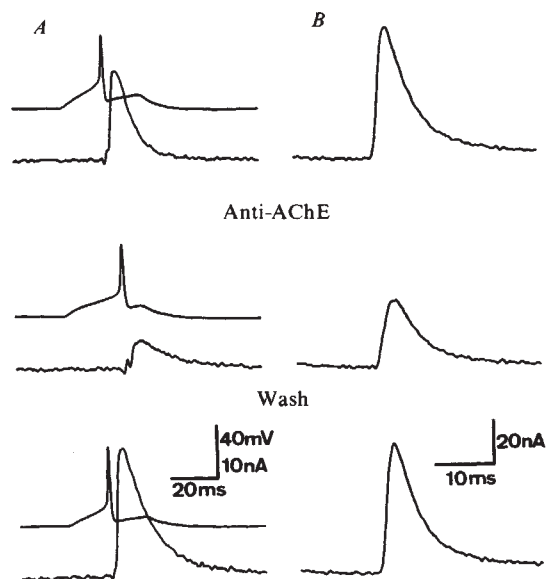


Fig. 2 Effects of phospholine on two cholinergic synapses. *A*, In the buccal ganglion, the inhibitory p.s.c. (lower traces) results from evoked spike by prolonged square pulse in the presynaptic cell (upper traces). The postsynaptic cell was voltage clamped at -80 mV. The postsynaptic response was potentiated after 10 min in phospholine and 30 min wash (bottom recordings). The decay time constant was increased from 9 (upper recordings) to 13 ms (bottom recordings). *B*, The excitatory postsynaptic current e.p.s.c. in R_{15} (clamped to -70 mV) results from threshold stimulation of the left pleuro-abdominal connective. The response, after phospholine was washed out, was unchanged (bottom trace) with respect to control (upper trace).

AChE inhibitors used were: phospholine at a concentration of 5×10^{-4} M, parathion (Serva) at 3×10^{-4} M, paraoxon (Serva) at 3×10^{-4} M, O-ethyl S-2-diisopropyl-aminoethyl methyl phosphonothioate (MPT) at 2×10^{-4} M and prostigmine at 10^{-5} M. Contrathion (pralidoxime sulfomethylate) at 10^{-3} M was used as a reactivator of AChE. Tetrodotoxin (10^{-4} M) was obtained from Sigma. Normal saline composition was: NaCl 460 mM, KCl 10 mM, CaCl_2 11 mM, MgCl_2 25 mM, MgSO_4 28 mM, Tris-HCl 10 mM, pH 7.8. The action of each AChE inhibitor was studied on at least 10 preparations (28 for phospholine) for both ACh and carbachol.

Current responses to applied ACh were depressed in the presence of organophosphate AChE inhibitors, probably due to a curare-like effect of these compounds. This effect was reversible after washing the preparation, as has been shown previously^{15,16}. After washing, the amplitude of the response increased up to 300% (mean $269 \pm 32\%$) of the test response (Fig. 1*A*). The current response to ACh application was also potentiated in the presence of prostigmine, a carbamate AChE inhibitor (Fig. 1*B*). The response to ACh ionophoresis was also facilitated in the presence of contrathion, 10^{-3} M, a reactivator of AChE poisoned by organophosphate compounds and a powerful rapidly reversible AChE inhibitor¹⁵.

The observed potentiations could be due to the inhibition of the ACh hydrolysing function of AChE, thus leading to an increase in available ACh. However, when carbachol was applied and the preparation submitted to the same treatments, the current responses were also potentiated, although to a smaller degree than for ACh (mean $176 \pm 7\%$) (Fig. 1*A*, *B*). The potentiation of the carbachol response cannot be due to an increase of available agonist because carbachol is not hydrolysed by AChE. Also, because there are no synapses on the soma, an indirect effect of AChE resulting in ACh leakage or activation of some presynaptic endings is unlikely (the potentiating effect is not seen on D-type receptor in a similar cellular environment; see below). Furthermore, carbachol was applied to the soma, which was freely exposed to the perfusing fluid,

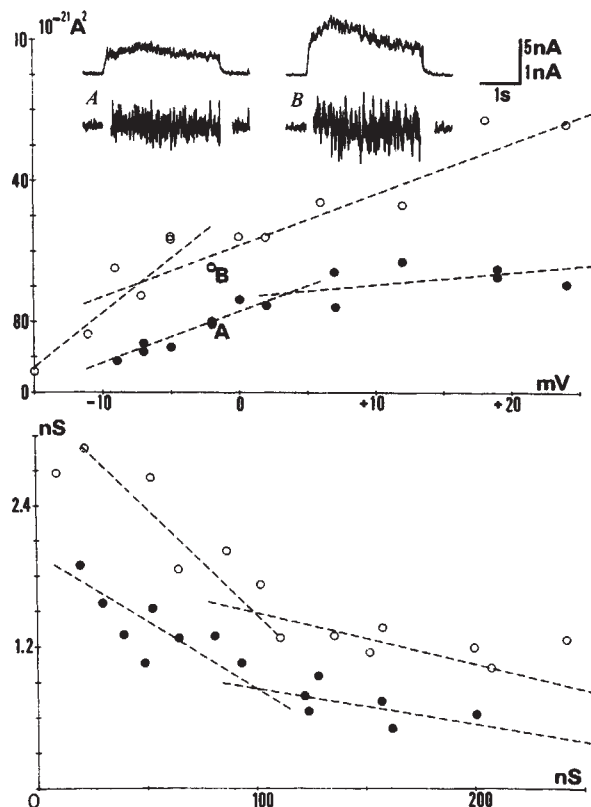


Fig. 3 Upper graph: variance (ordinate) versus presynaptic depolarization (abscissa) for the cholinergic synaptic couple in the buccal ganglion (solid circles, test; open circles, after phospholine 4×10^{-4} M treatment and wash). The pre- and postsynaptic cells were simultaneously voltage clamped at -50 mV and -80 mV, respectively. The preparation was bathed with TTX (Sigma), 10^{-4} M. The presynaptic neurone was depolarized for 3 s to different levels of membrane potential. The inserts show low-gain d.c. (upper traces) and high-gain a.c. (lower traces) current records (l.d.i.p.s.c.) obtained for the same depolarization (-2 mV) of the presynaptic cell before (*A*) and after (*B*) phospholine treatment. The increased mean current in *B* is totally due to the increase in the individual m.p.s.c. and not to a change in the number of quanta released. Lower graph: amplitude of m.p.s.c. expressed as conductance versus total mean conductance of the response before (●) and after (○) phospholine treatment. The m.p.s.c. showed a clear increase. For increasing membrane potential depolarizations above 0 mV, the relation variance/mean total current changed slope (upper graph), as has already been shown^{15,16}; but it seems significant that the percentage increase of m.p.s.c.s after application of anti-AChE drugs was about the same for a wide range of mean current values (lower graph). This suggests that in this range the individual m.p.s.c.s were independent of each other.

so that the distribution of concentrations of the drug over the injected region at different moments was probably identical whether AChE was active or inhibited.

The potentiation of the responses to ACh or carbachol application or to presynaptic stimulation was most probably linked to AChE inactivation. Indeed, it is known that AChE is present in *Aplysia* ganglia and that it is the most potent esterase¹⁷. Also, with organophosphorus AChE poisoning, all these responses returned to control levels when the preparation was perfused with the AChE reactivator contrathion for 10 min followed by prolonged washing. Furthermore, none of the observed changes could be attributed to a modification of the Cl^- reversal potential, as this was monitored throughout all experiments.

Thus, the potentiation of the responses could be attributed to a change in the properties of the ACh receptors, resulting in either a change in Cl^- channel characteristics or an increased number of activated AChRs. When the mean currents induced

by ACh and carbachol were identical, the single channel Cl^- conductance for channels opened by carbachol was greater than for those opened by ACh. With a mean response of 20 nS, the channel conductance calculated using Campbell's theorem was 3.4 ± 0.8 pS for ACh and 5.2 ± 1.1 pS for carbachol. The power density spectrum of the current noise produced by application of carbachol was shifted towards higher frequencies with respect to the ACh spectrum (Fig. 1C, a), indicating a reduction in the channel open time. Certainly, noise analysis has its limitations and must be interpreted with caution, especially when the noise power spectra of the Cl^- channel show two components^{12,14,18}. Thus, it may seem unwise to give absolute values for the channel parameters. However, the detection of relative changes was sufficient for our present purpose. Whatever the reason for these differences in calculated conductances and open times for ACh and carbachol after AChE inhibition (Fig. 1C, b), we found that the channel parameters were not changed significantly by organophosphorus compounds or prostigmine. Channel conductance was 3.5 ± 0.7 pS for ACh and 4.7 ± 1.0 pS for carbachol in eight experiments after AChE inhibition using phopholine.

Thus, as the channel characteristics were unmodified when AChE was inactivated, it seems likely that the potentiated response to carbachol following AChE inhibition was due to recruitment of an increased number of AChRs. This might be due to a change in receptor affinity so that more channels opened for a given dose of agonist.

As with sympathetic ganglionic synapses¹⁹, hydrolysis of ACh does not seem to be a limiting factor for the action of released ACh at the cholinergic synapse of the *Aplysia* buccal ganglion¹⁴. Thus, at these latter synapses one would not expect an increase in amplitude or duration of the evoked p.s.c. or of the m.p.s.c. after AChE inactivation. However, all AChE inhibitors increased the amplitude of both p.s.c. (Fig. 2A) and m.p.s.c. (Fig. 3) by 50% or more, as calculated from the l.d.i.p.s.c., and their duration was increased up to 50%. Analysis of the l.d.i.p.s.c. also indicated that the AChE inhibition did not change the number of quanta liberated per impulse.

The potentiating effects of AChE inhibitors were not observed in the R_{15} neurone, which contains D-type ACh receptor-opening, cationic, mostly sodium-selective channels^{6,20,21}. In this cell the response to the ionophoretic application of carbachol was not modified by any of the AChE inhibitors used (except for the washable curare-like depression in the presence of organophosphate compounds; middle trace, Fig. 2B). This absence of action not only indicated differences in functional receptor properties between R_{15} and the postsynaptic buccal ganglion neurones, but also could be considered as a good control: it shows that AChE inhibitors did not act non-specifically on the neuronal membrane and that the potentiation was not due to an experimental artefact such as local movements of the ionophoretic micropipettes.

The evidence indicates that at the cholinergic synapse in the buccal ganglion, AChE inhibitors have in some way modified the AChR molecule. It is possible that AChE inhibitors act directly on the AChR in two ways: through an easily reversible curare-like effect, or by causing a change in the receptor affinity for the agonist. A plausible alternative would be to consider that AChE inhibitors act indirectly on the AChR by inactivating AChE. This would require the existence of a molecular relationship between AChE and AChR and that AChE exerts an action on some receptor property of the AChR which modulates its readiness to be activated by ACh. Such an action would differ depending on whether AChE was in an active or inactive state. This hypothesis would explain why a similar facilitation of the agonist response is observed with AChE inhibitors of quite different molecular constitution (organophosphate compounds, carbamates, oximes). It seems unlikely that all the AChE inhibitors would directly modify the AChR in the same manner. Further, in the case of organophosphate compounds, the hypothesis would account for the restoration of the control situation with oximes. The absence of an effect on the R_{15}

D-receptor might then be due to a small density or absence of AChE or because the link here between AChE and AChR is weak. Our experiments were made at neuro-neuronal synapses and on somatic membranes where AChE is membrane-bound²² and is thus intercalated with AChRs in the same membrane. This situation is different from that for the neuromuscular junction, where the ultrastructural organization is distinct and more complex. However, an attempt to decide whether the facilitatory action of AChE inhibitors results from direct or indirect effects on the AChR seems premature. Nevertheless, it would be interesting to test for this effect at other synapses.

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Development of orientation columns in cat striate cortex revealed by 2-deoxyglucose autoradiography

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In the striate cortex of adult monkeys and cats, both electrophysiology^{1–3} and the 2-deoxyglucose autoradiographic technique of Sokoloff^{4–8} suggest that neurones are arranged in functional columns or slabs that run through the full thickness of the cortex, each column containing cells with a preference for a particular orientation of line or edge in the visual field. There is disagreement, however, concerning the organization of visual cortex in very young animals and the role of visual experience in cortical development. Orientation-selective neurones clearly exist in immature cat cortex, but reports differ on their frequency, angular selectivity and degree of columnar organization (see ref. 9 for review). We have used 2-deoxyglucose autoradiography to investigate the development of cat striate cortex. This technique reveals the spatial distribution of activity in populations of neurones and should therefore provide information about how the columnar pattern develops and whether its maturation depends on visual stimulation. We report here that in normal animals, periodic metabolic labelling around layer IV was first clearly observed at 21 days of age and by 35 days the pattern had become truly columnar; in a matched series of animals deprived of normal pattern vision no differential label was observed except for weak periodicity in a single 35-day-old animal. These results suggest that cat striate cortex is immature at the time of eye-opening and that visual experience is crucial for normal maturation.

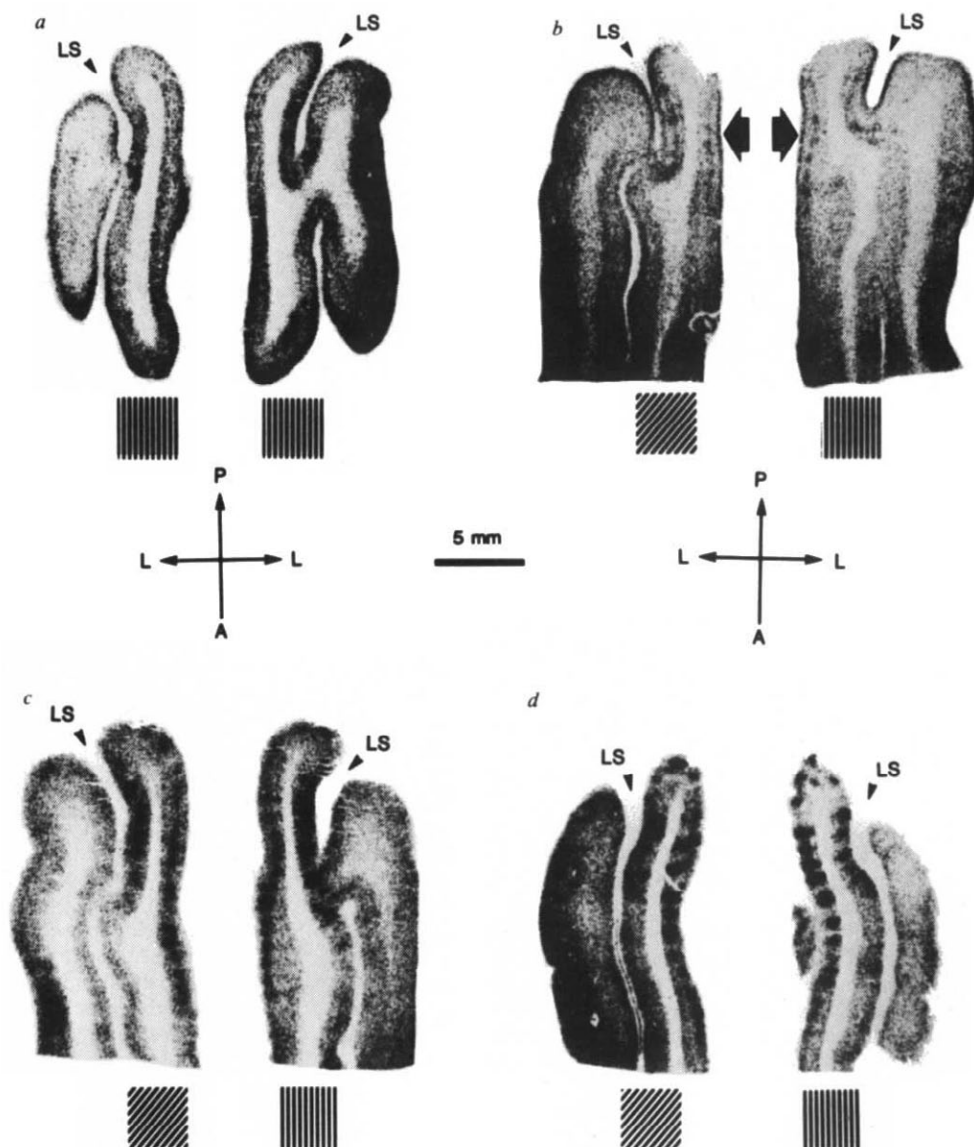
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2-Deoxyglucose autoradiography was performed on 13 cats aged 2–5 weeks. Initial preparation for 12 of the animals was similar to that described by Blakemore and Van Sluyters¹⁰. After premedication with atropine sulphate (60 µg per kg), the radial vein and trachea were cannulated under halothane and Althesin anaesthesia, respectively. All incisions were infiltrated with Tronothane (pramocaine chloride; Abbott). The animal was paralysed with gallamine triethiodide (10 mg per kg per h) and artificially hyperventilated with a 75% nitrous oxide–25% oxygen anaesthetic mixture. The electroencephalogram and electrocardiogram were monitored continuously, end-tidal P_{CO_2} was maintained between 4.5 and 5.0% and body temperature was held at 37.5 °C. One hour after paralysis, the eyes were refracted and the areae centrales plotted using a reversible ophthalmoscope. Visual stimulation was started when Althesin-induced spikes had disappeared from the electroencephalogram and the animal was judged to be in a steady state of light anaesthesia.

For the 12 anaesthetized paralysed animals, the left and right visual hemifields were stimulated respectively with oblique and vertical stripes generated on oscilloscope screens: the animal faced the two adjacent screens, each of which subtended 25° horizontally and 22° vertically, separated by a gap of 4°. The projections of the areae centrales were adjusted with prisms to

superimpose them on the centre of the mask separating the two screens. In some of the younger animals, the areae centrales were not clearly visible and prisms were used to adjust the separation of the projections of the optic disks to 30° to achieve approximate superimposition of the visual axes. The computer-generated visual stimulus consisted of drifting gratings of square-wave luminance profile (mean luminance ~250 cd m⁻²; contrast 0.75). The spatial frequencies used were 0.05, 0.2 and 0.8 c deg⁻¹, drifting at 1 or 2 Hz in either direction. Each spatiotemporal combination was presented for 10 s and successive stimuli, including a 10-s blank to counteract habituation of neurones, were presented pseudorandomly. The same stimulus was presented simultaneously on each screen except that the right-hand grating was vertical and the left-hand grating oblique, at 45°; 5 min after the onset of visual stimulation, 1.5 mCi per kg ³H-2-deoxyglucose (23 Ci mmol⁻¹; Amersham) was slowly infused intravenously. After 45 min, the animal was deeply anaesthetized with pentobarbitone sodium and perfused through the heart with the paraformaldehyde-based fixative of McLean and Nakane^{11,12}. The brain was removed, blocked and frozen slowly in isopentane at -70 °C. Horizontal sections of visual cortex (frozen in a cryostat) were cut at 20 µm, picked up on coverslips and dried rapidly on a hot plate. The sections were exposed for 4 weeks to LKB Ultrafilm which was then

Fig. 1 Montages of autoradiographs from normal cats demonstrating the development of orientation columns. *a*, Day 14 animal; *b*, day 21; *c*, day 28; *d*, day 35. Each montage was generated by superimposing photographic negatives made directly, at $\times 6$ magnification, from three adjacent autoradiographic images; contact prints were made from the superimposed negatives^{13,14}. For each animal, both hemispheres received identical processing after the initial visual stimulation. The autoradiographs show horizontal sections through the lateral and suprasylvian gyri, separated by the lateral sulcus (LS), of both hemispheres. Columnar label is apparent only in the posterior lateral gyrus and is restricted to grey matter; the white matter is unlabelled. In the 35-day animal columnar label extends through the depth of the cortex; regular periodicities in label first appear at 21 days in layer IV (arrowed). All animals except one had one hemisphere stimulated with vertical gratings and the other with oblique gratings as shown. The 14-day normal illustrated here received only vertical stimulation; it was unanaesthetized and unparalysed, simply being hand-held in front of a single display screen for 45 min after injection of 2-deoxyglucose. The absence of periodic label confirms the result obtained from a conventionally prepared paralysed and anaesthetized 14-day-old and suggests that anaesthesia is not limiting in these young animals.



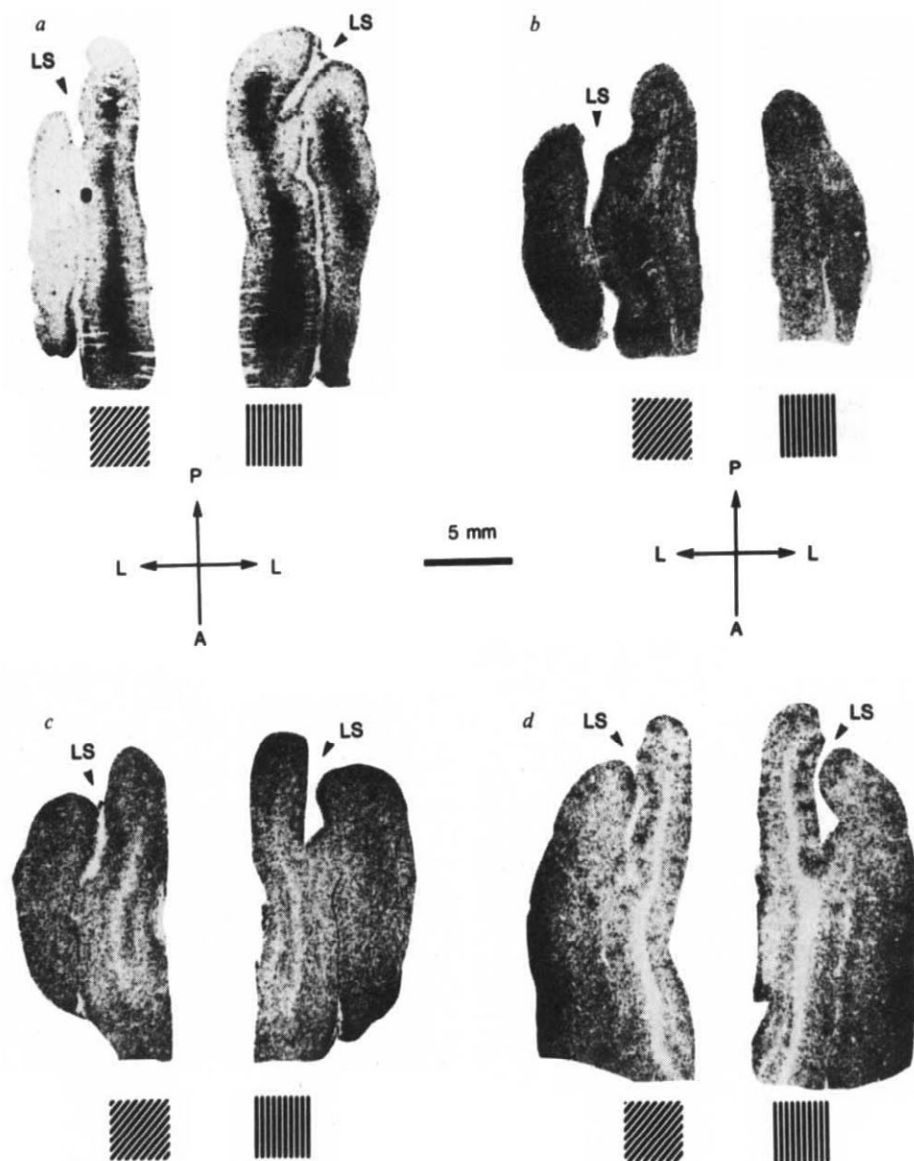


Fig. 2 Montages of autoradiographs from binocularly deprived cats of various ages demonstrating the failure of full development of orientation columns. *a*, Day 14 animal; *b*, day 21; *c*, day 28; *d*, day 35. Binocular deprivation was achieved by bilateral eyelid suture under ketamine anaesthesia before the time of normal eye-opening; animals which developed windows were discarded. The eyelids were opened during the preparative surgery, a maximum of 4 h before 2-deoxyglucose injection. During this period, visual experience was restricted with opaque masks placed in the visual field. The autoradiographs illustrate horizontal sections through lateral and suprasylvian gyri; the two hemispheres received visual stimulation of differing orientations as indicated. Differential cortical label was observed only in the oldest animal and was strongest in the lateral occipital pole. No foci of label were apparent in younger animals. The periodicities in *a* reflect cutting artefacts; the reason for the accumulation of label in the white matter of this animal is unclear.

developed in Kodak D19. Montages were generated from three adjacent autoradiographs and used to produce contact prints^{13,14}. All the sections illustrated are derived from such montages.

We studied seven normal cats (two at 14 days, one at 21 days, two at 28 days and two at 35 days old) for which Fig. 1 illustrates the maturation of the columnar pattern. Both 35-day-old animals showed a distinct pattern of labelling in the visual cortex (see Fig. 1*d*), similar to that described for the adult cat^{6-8,15}. The increased label in any one column extends through all cortical laminae, although it is strongest around layer IV. It is clearly periodic especially along the medial bank of the left striate cortex (stimulated by vertical gratings), with a periodicity of ~1.0 mm. The distribution of label in the younger animals was very different (Fig. 1*a-c*). In the 28-day-old animals, most of the increased 2-deoxyglucose uptake was located around layer IV although an increase in density of label extended into more superficial and deep laminae in some places, especially in the hemisphere stimulated by vertical gratings. The pattern is not as distinctly periodic as in the older animals, partly due to the reduced contrast of the differential labelling. Measurements on the left medial bank of visual cortex indicated a periodicity of 0.8–0.9 mm for label within layer IV. This pattern was seen in both normal 28-day-old cats, but at 21 days (Fig. 1*c*) differential label was not as distinct. In the montage shown

in Fig. 1*b*, small patches of denser label with a periodicity of ~1 mm can be seen in layer IV. This pattern was consistently present in both hemispheres but is better defined in the hemisphere stimulated by vertical gratings. In normal 14-day-old cats, no regularly periodic spots of differential label were apparent in striate cortex (Fig. 1*a*), although it is possible that subsequent quantitative analysis may reveal a weak underlying periodicity in grain density. Interestingly, patchy label is visible in Fig. 1*a*, in more lateral (non-striate) cortex; this was apparent only in the one unparalysed, unanaesthetized animal (illustrated) and not in another 14-day-old which was paralysed and anaesthetized.

The results in Fig. 1 show that the distinct adult orientation-dependent columnar pattern of metabolic activity arises gradually in cat visual cortex. To determine whether this reflects a purely passive maturational sequence or whether it is dependent on visual experience, we investigated six animals over the same age range (one at 14 days, one at 21 days, two at 28 days and two at 35 days) which had undergone bilateral eyelid suture before natural eye-opening. The results (Fig. 2) reveal a very different developmental history. Variation in the density of label was observed only in one of the oldest binocularly-deprived cats (35 days old, Fig. 2*d*). In the montage, slightly denser patches can just be distinguished in both hemispheres, most clearly in the lateral bank of the posterior pole of the left

occipital cortex. Here the increased labelling is not restricted entirely to layer IV but extends some distance superficially and has a periodicity of 1.0–1.1 mm. 2-Deoxyglucose autoradiography on binocularly deprived cats at earlier ages (Fig. 2a–c) produced no evidence of differential label. In the two 28-day-old animals no foci of activity could be detected even within layer IV. These results suggest that binocular deprivation severely disrupts the normal ontogenesis of orientation columns as defined by 2-deoxyglucose uptake.

It is clear from neurophysiological studies that a population of orientation-selective neurones does exist in the striate cortex of very young cats, even before 14 days old^{10,16}, but that the proportion of such neurones does not attain adult levels until 4–6 weeks old^{10,17,18}. Equally important, there is a gradual decrease in the tendency of neurones to habituate¹⁹ as well as an increase in responsiveness to the preferred stimulus. The optics of the cat eye also improves during this period but the poor optics is reported not to limit the orientation selectivity of striate neurones¹⁸. The appearance of clear differential 2-deoxyglucose uptake requires the presence of spatially localized populations of neurones that are both orientation-selective and more responsive to the preferred orientation than their non-selective neighbours. Habituation and poor responsiveness, combined with a substantial proportion of non-selective neurones, presumably explain the failure of the 2-deoxyglucose method to reveal clear periodicity of activity in the youngest animals studied here. However, the maturational changes in labelling in normal cats demonstrate absolutely that the pattern becomes more distinct with age, that it is virtually adult by 35 days, and that differential orientation-dependent activity when first evident is distributed around layer IV and is not fully columnar. Interestingly, Blakemore and Van Sluysers¹⁰, using physiological methods, found that most innately-specified neurones in very young animals, as well as in binocularly deprived cats, are located around layer IV.

The question of the role of visual experience in the development of striate cortex has been more controversial. Sherker and Stryker²⁰, in a quantitative study of binocularly deprived cats between 3 and 4 weeks old, reported numerous orientation-selective neurones with angular tuning similar to that in the adult cat. Other studies^{10,17,18} indicate that binocular deprivation prevents the full development of orientation selectivity. Our results suggest that binocular deprivation seriously impairs the development of orientation columns. We saw no obvious differential labelling pattern in deprived animals younger than 5 weeks and even then the distribution was irregular and poorly defined compared with that in normally reared animals. We believe this difference reflects changes in the selectivity rather than just the responsiveness of neurones as clear discontinuities of label are seen in binocularly deprived animals after monocular stimulation with many orientations, presumably corresponding to the pattern of ocular dominance columns (I.D.T., M.K. and C.B., unpublished observations). The sequence seen in binocularly deprived cats suggests that visual experience is crucial for maintaining the normal development of orientation columns.

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Assessment of chlorination by human neutrophils

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On phagocytosing a microorganism, the neutrophil (polymorphonuclear leukocyte, PMN) consumes oxygen at a sharply elevated rate¹. The oxygen is used to kill the microorganism, presumably being used to produce a potent oxidizing agent or agents. Candidates for these bactericidal agents are singlet oxygen, hydroxyl radical and chlorinating agents (that is, species containing 'active' Cl in a formal +1 oxidation state: HOCl, Cl₂, N-chloroamides, and so on)^{1–5}. We now report a semiquantitative assay for PMN-generated active chlorine based on its trapping with 1,3,5-trimethoxybenzene (TMB). Using this assay, we have found that at least 28% of the oxygen consumed by stimulated normal human PMNs is converted to active chlorinating agents.

It is generally agreed that oxygen respired by PMNs is first reduced to superoxide (O₂^{•−}) which is then converted to H₂O₂ and O₂ (refs 1–5 and Fig. 1). As neither O₂^{•−} nor H₂O₂ is strongly microbicidal, these species are thought to be precursors of more potent oxidizing agents. In the course of studies establishing that singlet oxygen is of minor importance in the microbicidal action, we found tentative evidence for the production of chlorinating agents by PMNs (refs 6, 7). A growing body of indirect evidence points to the same conclusion^{8–13}. Most importantly, PMNs contain myeloperoxidase, an enzyme that has been shown to catalyse the formation of hypochlorous acid (HOCl) from H₂O₂ and Cl[−] *in vitro*¹¹ (Fig. 1). In the phagocytic vacuole, HOCl could react further, forming secondary chlorinating agents (for example, Cl₂, N-chloroamines, N-chloroamides)⁹. Weiss *et al.*¹⁴ recently reported that human neutrophils generate chlorinating agents on being stimulated by opsonized zymosan or phorbol myristate acetate.

We now report an assay based on the reaction of TMB with chlorinating agents to yield a stable, characteristic product, 2-chloro-1,3,5-trimethoxybenzene (TMBCl)¹⁵ (Fig. 1). In a cell-free system, the reaction of TMB (10^{−4} M) with HOCl (10^{−4} M) was >90% complete after 30 s in albumin-free HEPES-buffered saline (pH 7.4, 37 °C). To detect very small amounts of TMB/TMBCl quantitatively, ¹⁴C-labelled TMB was prepared by methylating 3,5-dimethoxyphenol with ¹⁴C-methyl iodide¹⁶.

Using this reagent, chlorination (that is, TMBCl generation) by stimulated human PMNs was measured; the time course of its production is shown in Fig. 2. The identity of the chlorination product, TMBCl, was confirmed by gas chromatography–mass spectroscopy analysis (data not shown). No other products derived from TMB were detected. We performed a series of 19 chlorination assays, in which PMNs were incubated as described in Fig. 2 until they had consumed 75% of the available oxygen (20–30 min). In these experiments, the quantity of TMBCl formed was 28% (s.e.m. ± 2%) of the oxygen consumed. We regard this value as a lower limit for production of chlorinating agents, as TMB must compete with other reactive

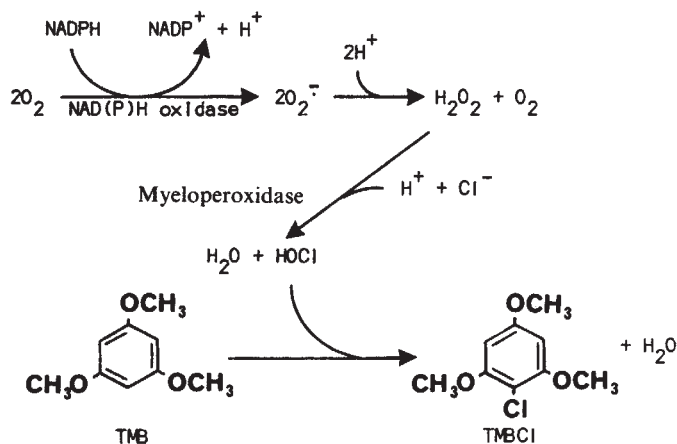


Fig. 1 Proposed scheme for HOCl production by human PMNs and its reaction with TMB.

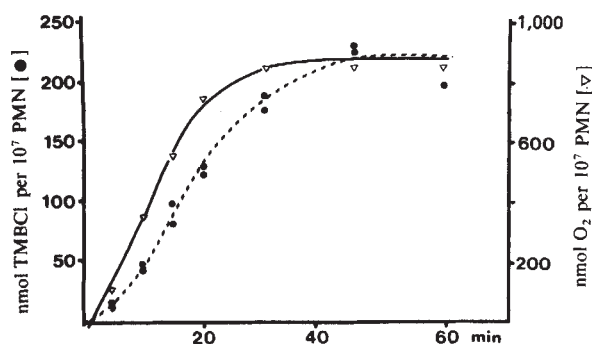


Fig. 2 Oxygen uptake and chlorination by normal human PMNs (2.5×10^6 cells per ml; prepared as previously described¹⁷, in HEPES-buffered saline (pH 7.4, 37°C) containing ^{14}C -TMB (1.0×10^{-4} M). The cells were stimulated with polystyrene beads (0.82 μm diameter; concentration = 1.1×10^9 beads ml^{-1}) containing adsorbed phorbol myristate acetate (50 ng per 10^{10} beads). Oxygen concentration was monitored with a Clark oxygen electrode. Chlorination was monitored by removing aliquots and measuring the concentration of TMB and TMBCl in each. Each aliquot was briefly sonicated to disrupt all cells, allowed to stand for 10 min (optional), treated with excess sodium thiosulphate and centrifuged at 13,000g for 2 min to pellet the polystyrene beads. The supernatant was passed through a Waters C₁₈ Sep Pac pre-column followed by 6 ml of water and 6 ml of methanol. A mixture of unlabelled standards (TMB and TMBCl) was added to the methanol fraction, which was evaporated under a stream of nitrogen at 35°C to a final volume of 200 μl . 20 μl of this solution was analysed by HPLC (Altex Ultrasphere ODS column, 4.6 $\times$ 250 mm) using isocratic elution with acetonitrile/water (45:55); UV absorption was monitored at 216 nm. The column effluent was divided using UV detection of the unlabelled standards as a guide and the ^{14}C -activity of each fraction was measured by liquid scintillation counting.

molecules (such as proteins) for the available chlorinating agents^{9,13}. Indeed, chlorination of TMB (10^{-4} M) with HOCl (10^{-4} M) was 95% suppressed in the presence of 5×10^{-6} M bovine serum albumin (BSA). As this concentration of BSA is also present in the PMN buffer medium, BSA almost certainly competes with TMB for some of the PMN-generated chlorinating agents. The fact that chlorination by PMNs is not more fully suppressed by BSA suggests that much of the observed chlorination may be occurring intracellularly.

Chlorination by PMNs from a patient with chronic granulomatous disease was also measured. Because of a genetic defect, such PMNs fail to exhibit a respiratory burst¹. Consistent with the scheme shown in Fig. 1, we could detect no (that is, ≤ 5 nmol per 10^7 cells) TMBCl production by these cells. Myeloperoxidase-deficient PMNs or azide-treated normal

PMNs are also deficient in their ability to chlorinate TMB (data not published).

These results demonstrate the formation of strong chlorinating agents by stimulated PMNs on a similar time scale to that of expression of microbicidal activity. As the precursors of the chlorinating agents (for example, MPO/H₂O₂/Cl⁻) appear to be localized in the phagocytic vacuole¹⁻⁵, our results support the argument¹⁻¹⁴ that chlorinating agents provide at least one strong microbicidal agent in the intact PMN. This new assay should prove generally useful for studying the metabolism and microbicidal function of normal and defective phagocytes.

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SB-restricted presentation of influenza and herpes simplex virus antigens to human T-lymphocyte clones

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The *HLA-D* region of the human major histocompatibility complex (MHC) has been shown to be homologous to the murine *I* region in terms of both structure and function. Both regions encode class II MHC molecules which restrict T-lymphocyte interactions with antigen-presenting cells. We have recently described the MHC restriction and antigen specificities of human T-lymphocyte clones directed at strain A influenza virus¹⁻³. The majority of T-lymphocyte clones recognized antigen in the context of cell surface interaction products encoded by *HLA-D/DR* genes. However, a few clones recognized antigen presented by cells histoincompatible for D/DR antigens. We report here that some of these clones recognize viral antigens in association with antigens encoded by genes identical with or closely linked to the recently described secondary B-cell (SB) locus of the MHC^{4,5}. This is the first report that SB-restricted antigen recognition may form an integral part of normal, human immune responses.

Peripheral blood lymphocytes (PBLs) from donor 144 (HLA-A1; B8; DR1, 3; Dw1, 3; SB2, 4) were primed *in vitro* with the strain A influenza virus A/Texas/1-77/x-49. Lymphoblasts were enriched on a Percoll gradient and cloned by limiting dilution in the presence of T-cell growth factor, antigen and irradiated autologous PBLs. Details of the cloning protocol, growth, phenotyping, antigen specificities and the behaviour of subclones have been described elsewhere^{1,2}. Assays for antigen presentation using histocompatible and histoincompatible PBLs required fractionation of the presenting cells into T (E⁺) and non-T (E⁻) fractions by rosetting with sheep erythrocytes treated with S-2-aminoethylisothiuronium bromide hydrobromide as described previously¹. Proliferation, as correlated with incorporation of tritiated methylthymidine, was assessed by liquid scintillation spectroscopy. Responses were categorized as positive or negative based on the kurtosis test for response outliers as described previously². In our system, T-lymphocyte clones do not respond to presenting cells in the absence of specific antigen^{1,2}.

The A/Texas neuraminidase-specific T-lymphocyte clone, FL1-71, was assayed for proliferation to influenza A virus

Table 1 The response to influenza virus segregates with SB2 on presenting cells

Antigen-presenting cell	D-region phenotype			Responder cell (c.p.m.)		
	DR	D	SB	FL1-71	CTLL A	CTLL B
1077*	3, 5	3, 5	4, 4	124	21,911	6,291
1072†	5, 4	5, DB3	2, 3	6,288	5,293	10,095
1003	3, 5	3, 5	4, 2	3,379	16,783	6,680
1074	5, 5	5, 5	4, 2	4,056	9,846	9,535
1078	3, 5	3, 5	4, 2	3,795	19,675	8,561
1073	3, 4	3, DB3	4, 3	48	18,566	5,526
144‡	1, 3	1, 3	2, 4	1,452	27,634	805
101	5, 6, 3	5, 6	2, 3	8,234	8,954	6,637
1023	1, 4	1, 10	3, 4	93	25,697	4,911
1022	2, 4	12, 4	6, 7	298	6,026	10,554

PBLs from donor 144 (HLA-A1; B8; DR1, 3; Dw1, 3; SB2, 4) were suspended at 5×10^5 per ml in RPMI 1640 medium containing 10% screened, pooled human A⁺ plasma, 2 mM L-glutamine, 25 mM HEPES buffer, 50 μ g ml⁻¹ gentamicin, 25 IU ml⁻¹ Na-heparin, 1 mM Na-pyruvate and 5 haemagglutinating units (HAU) per ml of influenza A/Texas/1-77/x-49 (Lot no. 53142; Merck, Sharpe and Dohme). After 6 days at 37 °C in 5% CO₂, responding lymphoblasts were cloned by limiting dilution at 0.3 cells per well in 60-well Terasaki microtitre plates; each well contained 20 μ l of medium as above and 20% T-cell growth factor (TCGF). TCGF was obtained from screened donors by stimulating irradiated PBLs with 0.1% v/v purified phytohaemagglutinin (PHA) for 48 h and collecting the supernatant. Generally, 10⁷ to 10⁹ cells can be obtained from each T-lymphocyte clone using described protocols¹. For assessment of antigen-specific stimulation, 5–10 $\times$ 10³ cloned cells and 5–10 $\times$ 10³ E⁻ antigen-presenting cells were added together in triplicate to 200 μ l of supplemented medium containing 5 HAU of virus per ml in 96-well, U-bottom plates. After incubation for 3 days each well was pulsed with 1 μ Ci of tritiated methylthymidine and incubated overnight. Cultures were collected onto glass fibre filters and the radiolabel incorporation was measured by liquid scintillation spectroscopy. The data are expressed as the mean of triplicate cultures. The standard error of each triplicate averaged less than 20% of the mean and is therefore not presented. Additionally, as no alloreactivity has ever been observed with this system, controls of the responding T-cell plus presenting cells without antigen have been omitted. The cell line CTLL A was derived from donor 144 primed *in vitro* to influenza as above and expanded in TCGF to provide a positive control. The cell line CTLL B was also derived using PBLs from donor 144 except that the cells were primed against a pool of irradiated allogeneic PBLs from five different donors before expansion in TCGF. Detailed descriptions of the cellular typing protocols used to detect HLA-D and SB antigens are found in refs 3 and 4.

* Father.

† Mother.

‡ Autologous control (TLC donor) and positive control for CTLL A and negative control for CTLL B.

presented by E⁻ cells from a family in which the SB2 locus was segregating (Table 1). Within this family, FL1-71 gave significant proliferation only in the presence of SB2⁺ presenting cells. The responses to antigen presented by SB2⁻ cells (1077 and 1073) were 12 ± 21 and 48 ± 14 c.p.m., respectively. In contrast, cells from donor 1077 and 1073 were able to present antigen to a non-clonal cell line reactive to influenza A (CTLL A) and stimulate an alloreactive cell line (CTLL B) sensitized to a pool of allogeneic donors. (Both these primed responder cell lines were derived from donor 144.) Thus the presenting cells from donors 1077 and 1073 failed to collaborate with the T-lymphocyte clone FL1-71 although they were functionally intact. Responses to autologous (144) presenting cells and an unrelated SB2⁺ individual are also shown in Table 1 as well as the lack of response to two SB2⁻ individuals, 1023 and 1022. Somewhat surprisingly, CTLL A appeared to recognize antigen in conjunction with presenting cells from donor 1022 even though there exists no apparent D-region compatibility (Table 1). However, one of the T-lymphocyte clones which has been previously described^{1,2} as DR1-restricted (FL1-6) also recognizes antigen presented by E⁻ cells from donor 1022; this result has been published elsewhere⁶. We postulate that 1022 shares an epitope with DR1⁺ individuals that is undetectable by conventional means or, more likely, that recombination within the D region in donor 1022 has occurred. Both alternatives are under investigation. These data suggest that donors 144 and 1022 share some common presenting element, but such a finding does not alter the results suggesting that FL1-71 may be restricted by an antigen associated with SB2.

Although no recombinant families with cross-overs isolating SB2 from D/DR are available, the statistical data from unrelated cell panels indicate an extremely tight association between SB2 and positive responses by FL1-71 ($P < 3 \times 10^{-4}$ by Fisher's

Table 2 Contingency analysis of associations between FL1-71 responses and various D-region antigens

Specificity of presenting cells	FL1-71/antigen				Fisher's exact probability	Corrected probability ($n = 19$)
	+/+	+/-	-/+	-/-		
DR1	2	3	5	6	0.635	NS
DR2	0	5	1	10	0.688	NS
DR3	0	5	5	6	0.106	NS
DR4	2	3	3	8	0.861	NS
DR5	2	3	2	9	0.937	NS
Dw6	1	4	2	9	0.786	NS
DR7	0	5	0	11	1.0	NS
DRw8	0	5	1	10	0.688	NS
Dw9	0	5	1	10	0.688	NS
Dw10	0	5	1	10	0.688	NS
Dw11	0	5	0	11	1.0	NS
Dw12	0	5	1	10	0.688	NS
SB1	0	5	4	7	0.181	NS
SB2	5	0	0	11	2.289×10^{-4}	4.349×10^{-3}
SB3	2	3	2	9	0.937	NS
SB4	3	2	10	1	0.214	NS
SB5	0	5	0	11	1.0	NS
SB6	0	5	1	10	0.688	NS
SB7	0	5	1	10	0.688	NS

In panel experiments, E⁻ antigen-presenting cells from 16 unrelated donors and the autologous control (144; HLA-A1; B8; DR1, 3; Dw1, 3; SB2, 4) were tested for their capacity to present influenza A/Texas/1-77/x-49 to the T-lymphocyte clone FL1-71. Proliferation assays were performed as described in the legend to Table 1. Representative data can also be seen in Table 1. Responses were classified as positive or negative based on a test for outliers using the kurtosis statistic as described previously². The data were divided into positive and negative responses and the results were analysed in a 2 $\times$ 2 contingency table and the probability of association (P) between D-region alleles and positive responses by FL1-71 was computed using Fisher's exact test. Although only a small panel has been tested, the results with SB2 are highly significant but they do not prove identity between SB2 and the interaction product recognized by the clone. NS, not significant.

Table 3 Responses of an HSV-specific, SB-restricted T-lymphocyte clone

Donor	Phenotype			Proliferative response (c.p.m.)	
	DR	D	SB	A53 + media	A53 + HSV
1053	3, 7	3, 7	1, ?	49	55
101	5, 6, 3	5, ?	2, 3	78	36
1022	4, 12	4, 2	6, 7	1,460	1,057
1199	3, ?	3, 5	1, 4	468	21,287
1003	3, 5	3, 5	2, 4	90	19,837
144*	1, 3	1, 3	2, 4	47	4,616
1011	1, ?	1, 4	2, 4	24	12,915
1023	1, 4	1, 10	3, 4	51	8,663
1005	2, 7	2, 7	4, ?	21	14,204
506	4, 6, 1	4, ?	4, ?	159	18,581
694	1, 5	ND	4, ?	151	19,385
1009	2, 7	2, 7	4, ?	47	19,120
9999	ND	ND	4, ?	191	15,041

PBLs from donor 144 (HLA-A1; B8; DR1, 3; Dw1, 3; SB2, 4) were primed to UV-inactivated lysates of HSV-infected VERO cells and cloned in a protocol as described in Table 1 legend. For assay, 10^4 of the cloned cells were added to 10^4 irradiated E⁻ antigen-presenting cells from 13 unrelated donors (including the autologous control, 144) who were previously typed for HLA and SB antigens. Culture conditions and assay procedures were identical to those described for the influenza system. Clone A53 gave positive responses only in the presence of antigen and E⁻ cells bearing SB4. The presenting cell, 1022, appeared to be stimulatory even in the absence of antigen and was therefore considered negative relative to other cells giving specific responses.

ND, not done.

* Autologous control (TLC donor).

exact test; Table 2). Even if this value is corrected by the number of antigenic specificities typed for ($n = 19$), the result remains highly significant.

In another system, T-lymphocyte clones were generated from the same donor (144) against herpes simplex virus (HSV) and one clone was identified (A53) which appeared restricted by SB4 (Table 3); essentially background level responses were obtained when SB4⁻ antigen-presenting cells were used to present HSV to A53. Such data from two different antigen systems confirm the generalization that the SB region may indeed encode functional interaction products. However, even though such data reveal a high degree of association they do not, at this stage, prove identity between SB2 and the interaction product recognized by the clone.

At present, there is no apparent correlation between restriction specificities and the antigen specificity or function of the T-lymphocyte clones. However, further experiments are required to define the complete functional capabilities of SB-region products. Although a clear functional dichotomy exists for molecules encoded by I-A and I-E in the mouse, this is not the case for SB and DR which both appear to code for I-E-like α and β chains⁷. Other data from our laboratory suggest that both D/DR and SB may comprise gene clusters encoding multiple cell-surface, class II molecules^{6,8}. Furthermore, it is not yet known whether such products can associate on the cell surface to form functional interaction products or complex alloantigens as has been described for the I-A⁸ and I-B⁹ chains⁹. We are now using T-lymphocyte clones to dissect the human MHC to investigate the role of different class II alloantigens in controlling immune responses to defined antigenic determinants. This approach is based on the hypothesis that multiple D-region products, probably encoded by 'immune response' genes, interact to control the response of any given T cell to particular antigenic epitopes.

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An inherited polymorphism in the human apolipoprotein A-I gene locus related to the development of atherosclerosis

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Epidemiological studies have identified elevated low density lipoprotein (LDL)¹⁻³ and diminished high density lipoprotein (HDL) cholesterol levels^{3,4} as risk factors for coronary artery disease. The major protein component of HDL is apoprotein A-I (apo A-I), a polypeptide of 243 amino acids of known primary amino acid sequence⁵. This apoprotein serves as a cofactor for the plasma lecithin-cholesterol acyltransferase (LCAT) enzyme responsible for the formation of most cholesteryl esters in plasma, and also promotes cholesterol efflux from cells^{6,7}. The primary translation product of apo A-I contains both a pre and a pro segment⁸, and post-translational processing of apo A-I may be involved in the formation of the functional plasma apo A-I isoproteins^{9,10}. Defective apo A-I processing may be the underlying problem in Tangier disease¹¹, in which patients have low plasma HDL and apo A-I levels despite normal apo A-I synthesis^{12,13}. Patients have been reported with conditions distinct from Tangier disease in whom severe deficiency or absence of apo A-I has been associated with very low HDL levels and severe coronary artery disease^{14,15}. We have now examined the apo A-I gene in two such patients and their first degree relatives. These patients have been reported to have skin and tendon xanthomas, corneal clouding and severe premature coronary atherosclerosis associated with very low HDL levels and deficiencies of two apoproteins, apo A-I and apo C-III¹⁵. We show that both probands are homozygous for a defect in the apo A-I gene locus.

We have previously reported the isolation of apo A-I double-stranded (ds) cDNA clones from an adult human liver cDNA library¹⁶. One of these clones (pAI-113) carries an insert having a DNA sequence corresponding to the 3'-untranslated region and extending in the 5' direction to the codon representing the 94th amino acid of apo A-I. This clone was used to prepare the apo A-I probe for the experiments described here. Figure 1 shows the hybridization patterns obtained by restriction digestions of chromosomal DNA prepared from a normal individual and from one of the apo A-I-deficient patients after blotting¹⁷ and hybridization with the apo A-I probe. In the normal individual the probe hybridized to a unique 13-kilobase (kb) *EcoRI* fragment, whereas *EcoRI*-digested DNA from the patient hybridized to a unique 6.5-kb band. To examine further the molecular basis of this difference, we performed genomic blot analysis of normal individuals and both probands showing apo A-I deficiency using various combinations of restriction enzymes (Fig. 1). The sizes of the DNA fragments hybridizing to the apo A-I probe are shown in Fig. 1 and have been compiled in Table 1. As can be seen, *EcoRI* and *PstI* either singly or in combination with other enzymes produce fragments of different size when DNA from normal individuals and apo A-I-deficient

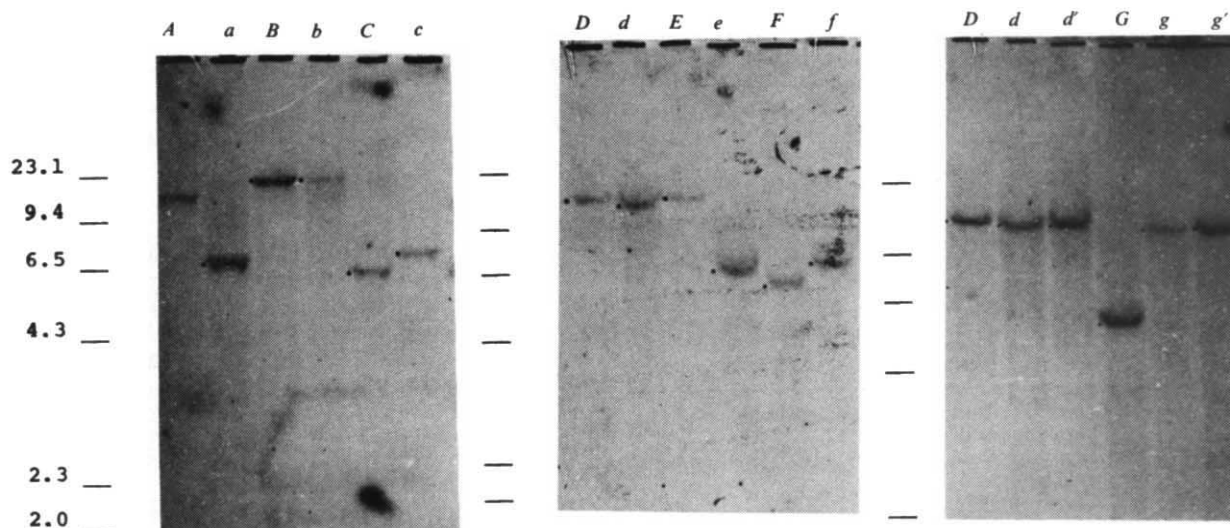
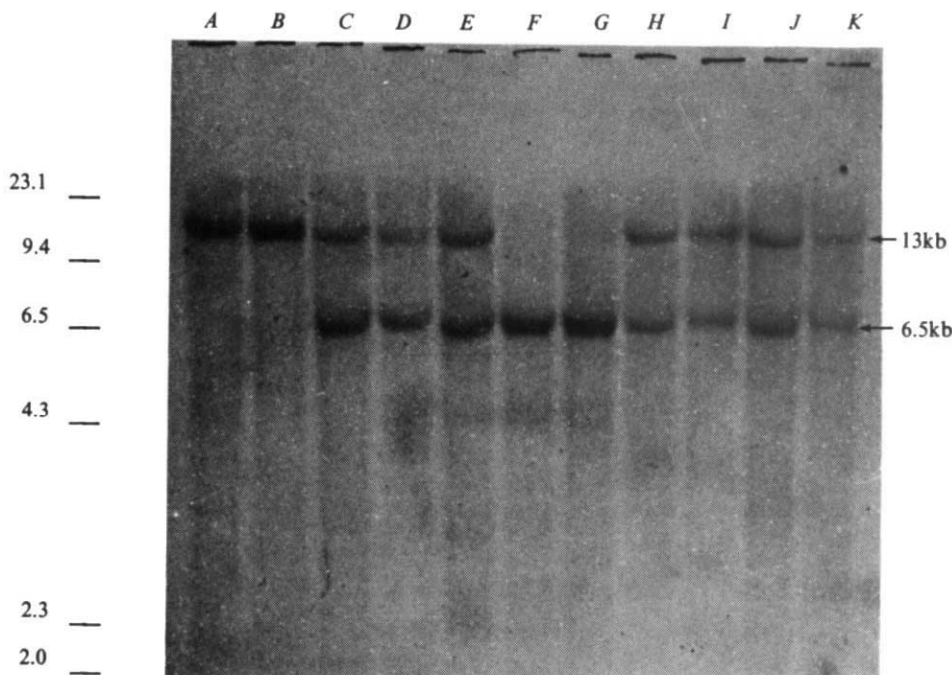


Fig. 1 Southern blots of DNA from normal and apo A-I-deficient individuals (upper and lower case letters respectively) digested with *Eco*RI (A, a), *Hind*III (B, b), *Eco*RI + *Hind*III (C, c), *Bam*HI (D, d, d'), *Bam*HI + *Eco*RI (E, e), *Bam*HI + *Eco*RI + *Hind*III (F, f), *Bam*HI + *Hind*III (G, g, g'). Lymphocytes were isolated from peripheral blood¹⁹ and used as the source of DNA. The DNA was prepared by resuspending the lymphocytes in 0.5M EDTA, pH 8 and adding SDS and proteinase K to final concentrations of 0.5% and 100 μ g ml⁻¹, respectively. After incubation at 55 °C for 2–3 h, the sample was extracted with phenol–chloroform, concentrated with secondary butanol, ethanol-precipitated, resuspended in TE (10mM Tris-HCl, 1 mM EDTA pH 7.5), dialysed against TE, and stored at 4 °C. The DNA was digested with restriction enzymes, electrophoresed in 0.8% agarose and transferred to nitrocellulose filters¹⁷. The filters were prehybridized at 65 °C in 10×Denardt's solution, 5×SET²⁰, 0.1 M Na-phosphate pH 7, 0.1% Na-pyrophosphate, 0.1% SDS, and 30 μ g ml⁻¹ heat-denatured salmon sperm DNA. The hybridization mixture was identical to the prehybridization solution except that it contained radiolabelled heat-denatured probe (2×10⁶ c.p.m. ml⁻¹). The hybridization probe was the insert of clone pAI-113 excised with *Pst*I and labelled with ³²P by nick-translation. After hybridization for 15–20 h at 65 °C, the excess probe was washed off by incubation in 0.1×SSC¹⁶ and 0.1% SDS for 1 h at 65 °C. X-ray film was sandwiched between the filters and an intensification screen and exposed at –70 °C for 3 days.

Fig. 2 Southern blot of *Eco*RI-digested DNA from a normal individual (A) and from the apo A-I-deficient probands (F) and (G). Blots are also shown for the maternal grandfather (B), father (C), mother (D) and brother (E) of the probands. In addition, the son (H) and daughter (I) of proband (G), and the son (J) and daughter (K) of proband (F) are also shown. The hybridization conditions were as described in Fig. 1 legend.



patients is compared. These results (Table 1) indicate that a single restriction site polymorphism is not sufficient to explain the differences observed in the hybridization pattern.

Analysis of the chromosomal apo A-I gene isolated from a human fetal liver genomic library has positioned a *Hind*III site 100 base pairs (bp) upstream from the codon corresponding to the amino-terminus of the mature apo A-I protein (S.K.K., V.I.Z. and J.L.B., unpublished data and similar results in ref. 18). The DNA sequence corresponding to the apo A-I cDNA probe used in these experiments spans a region between 1,500 and 2,000 bp downstream from this *Hind*III site. In the case of the chromosomal DNA from the patients, the defect does not affect the hybridization of the apo A-I cDNA probe to

genomic DNA fragments but does interfere with the *Hind*III site. Thus it can be concluded that this defect involves the DNA sequence between the *Hind*III site and the sequence corresponding to the apo A-I cDNA probe. Isolation and analysis of the apo A-I gene from the apo A-I-deficient probands will be necessary to define the boundaries of this defect within the apo A-I gene locus region.

We have also examined DNA from the first degree relatives of the apo A-I-deficient patients who are asymptomatic but who have intermediate levels of HDL, apo A-I¹⁵ and apo C-III (R.A.N. and P. Alaupovic, unpublished data). Figure 2 shows the pattern obtained by *Eco*RI digestion of chromosomal DNA from these individuals after hybridization with the probe. In

Table 1 Size (in kb) of genomic restriction fragments hybridizing to the apo A-I-113 probe estimated from Fig. 1

	Normal individual	apo A-I-deficient individual
<i>EcoRI</i>	13	6.5
<i>BamHI</i>	12	11
<i>HindIII</i>	15*	15*
<i>PstI</i> †	2.2	0.95
<i>EcoRI</i> + <i>BamHI</i>	12	6.5
<i>EcoRI</i> + <i>HindIII</i>	5.5	6.5
<i>BamHI</i> + <i>HindIII</i>	5.5	11
<i>PstI</i> + <i>HindIII</i> †	1.7	0.95
<i>EcoRI</i> + <i>BamHI</i> + <i>HindIII</i>	5.5	6.5

* The length of the *HindIII* fragments is approximate as the gel used in Fig. 1 has a limited resolution in this size range.

† The sizes of these restriction fragments were estimated from another genomic blot (data not shown).

each of the first degree relatives, a 13-kb and a 6.5-kb band are revealed which appear to correspond to the wild type and the mutant apo A-I alleles, respectively. These family studies indicate that both affected sisters are homozygous for the same mutant allele and that the disease is transmitted in a typical autosomal recessive mendelian pattern. Similar analysis of 10 normal individuals and the husbands of the apo A-I-deficient probands showed that only the unique 13-kb *EcoRI* genomic fragment hybridizes to the apo A-I probe. Although the parents of the afflicted individuals are not known to be related, both are descended from ancestors of Scottish origin who lived in the Appalachian mountain region of southeastern Kentucky. Therefore, it was not surprising to find them both carrying what appears to be the same mutant allele.

It is of interest that the obligate heterozygotes in this family all had diminished HDL levels in the range associated with an increased risk of coronary disease. Thus, carriage of this mutant apo A-I allele may be an identifiable genetic cause of low HDL levels. Whether this mutation is a common cause of low HDL levels in the general population can only be determined by a study of a population having low HDL levels for this altered apo A-I allele. In addition, the ability to unequivocally identify heterozygotes for this condition may be beneficial in genetic counselling the offspring and brother of these affected sisters. Furthermore, should these individuals marry carriers of the same mutant apo A-I gene, it would be possible through pre-natal diagnosis to detect homozygous offspring.

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An idiotypic determinant formed by both immunoglobulin constant and variable regions

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Immunoglobulin idiotypes are serologically defined determinants associated with the variable (V) region of antibody molecules (reviewed in refs 1-4). One of the best defined idotype systems is that borne by the phosphorylcholine (PC)-binding IgA proteins TEPC15 (T15) and HOPC8 (H8)⁵. The T15 idotype, defined by sera raised in A strain mice⁶, or in rabbits⁷, is considered identical to that expressed by the majority of BALB/c anti-PC antibodies. To define the idiotype determinants (idiotopes) of which the T15 idotype is comprised, monoclonal anti-T15 antibodies were used here to examine both serum and monoclonal anti-PC antibodies. The latter were found to differ from T15 with respect to the idiope defined by the monoclonal anti-idiotope antibody, 21A5, in that the '21A5 idiotope' was absent from anti-PC sera; of the monoclonal anti-PC antibodies examined, only those which were both T15⁺ and of the IgA isotype seemed to express this idiotope fully. This result suggests that not only the V region, but also the constant (C) region, of the immunoglobulin molecule can contribute to the formation of an idiotype determinant. Isotype-restricted idiotopes may be involved in the regulation of antibody responses of particular classes.

Although the production and characterization of the monoclonal anti-T15 idiotope antibodies 21A5 and 23B3 will be described in detail elsewhere (ref. 8 and manuscript in preparation), the idiotype specificity of these antibodies is shown in Table 1. Binding of ¹²⁵I-labelled H8 (¹²⁵I-H8) to either 21A5 or 23B3 was inhibited by the addition of H8, but not by MOPC 167, an IgA PC-binding myeloma protein (PCBMP) which lacks the T15 idiotpe⁵. However, only the binding of 23B3 is hapten-inhibitable: free hapten (PC chloride) or PC-conjugated⁹ bovine serum albumin (BSA) prevented the interaction of this antibody with its idiotpe. There was no significant inhibition of the

Table 1 Specificity of monoclonal anti-idiotpe antibodies, 21A5 and 23B3

Competitor	Maximum inhibition (%)		Amount required for 50% inhibition	
	21A5	23B3	21A5	23B3
HOPC 8	99	96	25 ng	43 ng
MOPC 167	3	31	> 5,000 ng	> 5,000 ng
Phosphorylcholine chloride	—	72	> 50 nmol	0.4 nmol
PC-BSA	11	86	> 5,000 ng	63 ng

¹²⁵I-H8 was mixed with appropriate dilutions of unlabelled competitor and added to wells of a polyvinylchloride microtitre tray (Dynatech) that had previously been coated with a limiting amount of purified 21A5 or 23B3 immunoglobulin. Following an overnight incubation, the trays were washed thoroughly, cut and individual wells counted in a Packard γ counter. Inhibition caused by each dilution of competitor (tested in triplicate or quadruplicate) was calculated relative to a set of wells which had received ¹²⁵I-H8 alone. The amount of competitor required for 50% inhibition of the binding of ¹²⁵I-H8 to the solid phase was estimated from interpolation of the data. Phosphorylcholine chloride (Sigma) was diluted from 10⁻³ M in borate-buffered saline, pH 8.4, containing 1% BSA (Armour) and 0.05% 'Tween-20' (Sigma). Other competitors were diluted from 100 μg ml⁻¹ in phosphate-buffered saline, pH 7.3, containing 1% BSA, 10 μg ml⁻¹ PC-absorbed normal A/J IgG and 0.05% 'Tween-20'. PC-BSA was prepared as described by Gearhart et al.⁹.

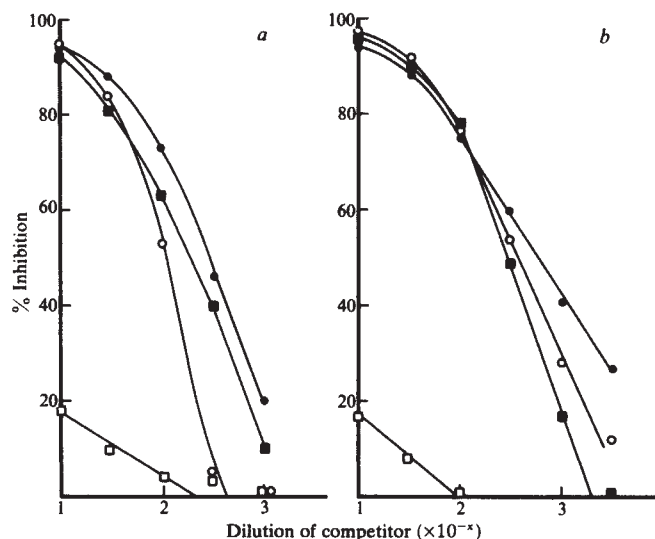


Fig. 1 The 21A5 idiotope is not detected on serum anti-PC antibodies by competitive radioimmunoassay. Dilutions of either H8 (○, ●) or BALB/c anti-PC serum (□, ■) were added to wells coated with monoclonal anti-T15 antibodies 21A5 (open symbols) or 23B3 (closed symbols). Other details of the assay are described in Table 1 legend. *a*, Sera obtained from BALB/c mice 7 days after immunization with 100 µg PC-KLH in complete Freund's adjuvant (Difco). *b*, Sera obtained from the same mice, boosted with 100 µg PC-KLH in incomplete Freund's adjuvant (Difco) on day 10 and bled 4 weeks later.

binding of ^{125}I -H8 to 21A5 even at the highest concentration of hapten tested. These results suggest that the determinant with which 21A5 reacts is not associated with the antigen-binding site of the H8 molecule.

To examine the expression of the 21A5 and 23B3 idiotopes on serum antibodies, BALB/c mice were immunized with PC-keyhole limpet haemocyanin (KLH); the sera, collected after 7 days and after 5 weeks, were tested by competitive radioimmunoassay (Fig. 1). Both the 21A5 and 23B3 idiotopes were present below detectable levels ($< 1 \mu\text{g ml}^{-1}$) in normal mouse serum, nor was there a significant concentration of anti-PC antibodies in such sera (data not shown). As the mice used in these experiments were from specific pathogen-free colonies, they would have had minimal exposure to environmental PC and may therefore have been expected to contain very low levels of naturally occurring anti-PC antibodies (see ref. 6). Although the 23B3 idiotope was readily detectable in the immune sera, no significant inhibition of the interaction between 21A5 and ^{125}I -H8 was observed in either the primary or the secondary serum pool. Although these results suggest that the 21A5 idiotope is not present among BALB/c anti-PC antibodies, this interpretation seemed unlikely in view of previous reports of the idiotypic identity¹⁰ and N-terminal amino acid sequence homology¹¹ between BALB/c anti-PC antibodies and T15. For this reason, and also because the 21A5 idiotope has been demonstrated on three independent T15⁺ PCBMP (T15, H8 and S107; see Table 2), it is unlikely that the 21A5 idiotope is a 'private' idiotypic specificity unique to T15. The observation that the 21A5 idiotope was present on three independent IgA PCBMP, but not in sera which may be expected to have low amounts of IgA antibodies, suggests that the 21A5 idiotope is associated with IgA antibodies.

A more rigorous demonstration of this association is shown in Table 2. Monoclonal anti-PC antibodies of the IgG, IgM and IgA isotypes were examined for their ability to inhibit the binding of 21A5 to ^{125}I -T15. The results show that approximately 16–50 ng of T15⁺ IgA was required for 50% inhibition in this assay, whereas no significant inhibition was caused by any of the T15⁺ monoclonal antibodies of the IgG or IgM isotypes. Of 2 T15⁺ IgG and 11 T15⁺ IgM monoclonal anti-PC antibodies tested, all showed this apparent lack of the 21A5 idiotope. Idiotypically cross-reactive (T15^{CR}) or T15⁻ IgG and

Table 2 The 21A5 idiotope is expressed predominantly on T15⁺ antibodies of the IgA isotype

Source of monoclonal anti-PC antibody	Isotype	T15 idiotype	Maximum inhibition (%)	Amount required for 50% inhibition (ng)
T15	IgA	+	90	50
H8	IgA	+	95	25
S107	IgA	+	92	40
CBBPC3	IgA	+	92	16
HPC 7.41.9*	IgA	CR	95	25
M167, M511, M603	IgA	—	<10	>5,000
HPCI 1-8*; 2L, 13D, 32D†	IgM	+	<10	ND
HPC5*; 1B†	IgM	CR	<10	ND
HPC4, 10, 16, 19, 104, 312*; 3C†	IgM	—	<10	ND
37I†	IgG1	+	<10	ND
HPC 9*	IgG1	CR	<10	ND
40C†	IgG1	—	<10	ND
HPC320*	IgG2b	+	<10	ND
HPC8, HPC50*; 5G†	IgG3	CR	<10	ND

PCBMP were purified (from ascites fluid of BALB/c mice bearing the appropriate myeloma) and tested for the 21A5 idiotope as described in Table 1 legend; maximum inhibition was determined for each PCBMP at a concentration of $10 \mu\text{g ml}^{-1}$. Hybridomas secreting anti-PC antibodies were produced from BALB/c mice immunized with either pneumococcal polysaccharide (*) (ref. 23) or PC-KLH (†). Supernatants were mixed with equal volumes of either ^{125}I -T15 (*) or ^{125}I -H8 (†) and tested for the expression of the 21A5 idiotope (as described in Table 1) and for the T15 idiotype by competitive radioimmunoassay using a panel of monoclonal anti-T15 antibodies. The inhibition of ligand binding to each of the panel of anti-idiotypic antibodies caused by a test supernatant was used to define the T15 idiotype as follows: T15⁺, >90% inhibition of binding to each anti-idiotypic antibody; T15^{CR}, intermediate inhibition; T15⁻, <10% inhibition. CR, cross-reactive. ND, not determined beyond the highest concentration tested, that is, a 1:2 dilution of hybridoma supernatant ($\sim 10 \mu\text{g ml}^{-1}$ antibody).

IgM antibodies were similarly ineffective competitors in this assay. However, the T15^{CR} IgA antibodies produced by the hybrid HPC 7.41.9 fully expressed the 21A5 idiotope. T15⁻ IgA PCBMP caused no inhibition, indicating the specificity of this assay. Thus, the 21A5 idiotope appears restricted to those antibodies of the IgA isotype which express the T15 idiotope.

There have been previous reports of apparent isotype restriction of idiotypes^{12,13}. For example, the cross-reactive idiotope on A/J anti-azobenzenearsonate antibodies was thought to be limited to the IgG1 class¹², but monoclonal antibodies of other isotypes have since been described which bear this idiotope^{14,15}. Different idiotypes were found on IgG3 and IgG1 antibodies raised against dinitrophenylated carriers: T-independent type 2 and T-dependent antigens, respectively¹³. Such idiotypes may reflect certain V_H - C_H associations, together with the selection of different precursors in response to these different antigens. The present study differs in that there seems to be an actual contribution of the α heavy chain to the formation of an idiotypic determinant, rather than the selection, among precursors of IgA-secreting cells, of those clones which express '21A5 V genes'. This contention is supported by the demonstrations of idiotypic identity between T15 and antibody-secreting cells^{16,17}. Stronger evidence is that the V_H , D and J_H (ref. 18), as well as the V_L and J_L (ref. 19) genes of T15 contain the germ-line sequence, which probably also encodes all BALB/c T15⁺ antibodies^{19,20}. As T15 contains the germ-line sequence and also the 21A5 idiotope, antibodies which lack this idiotope must have sequences which differ from that of T15 and the germ line. It is unlikely that anti-PC antibodies lacking the 21A5 idiotope (Fig. 1, Table 2) are all encoded by genes which have deviated from the germ-line sequence; a role for somatic mutation in generating the 21A5 idiotope can therefore be ruled out. The obvious difference between these antibodies and

the T15⁺ PCBMP is in their heavy-chain constant regions; the evidence suggests that it is this difference which is crucial to the formation of the 21A5 idiotope.

There are two possible ways in which the C_α chain can contribute to the formation of an idiotope. Folding of the C_α domains may result in a slightly different tertiary structure in the V region. Alternatively, some amino acids encoded by the C_α gene, together with V region amino acids (possibly encoded by the D segment), may directly form an idiotypic determinant. X-ray crystallography of another PCBMP, McPC 603, showed that the V_H domain closely approaches and interacts with the first domain of the constant region²¹. This region of the T15 molecule is therefore a candidate for the site of the 21A5 idiotope.

Immunoglobulin C_H regions contribute to antibody diversity insofar as they confer different biological effector functions on antibody molecules and, as illustrated by our present study, by the formation of isotype-specific idiotopes. A possible biological role for such idiotopes is in the control of antibody responses of a particular class. As postulated by Jerne²², antibodies such

as 21A5 could directly regulate the anti-PC response. More significantly, such idiotopes may be recognition structures for regulatory T cells which could either help or suppress cells producing antibody of a particular class. This establishes a framework for the simultaneous control of antigen specificity and biological effector function of an antibody response.

Monoclonal anti-idiotope antibodies allow the definition of individual idiotypic determinants, including those whose existence was unsuspected from studies based on anti-idiotypic sera. In view of the potential biological importance of isotype-restricted idiotopes, we predict that more of these idiotopes will be identified as more monoclonal anti-idiotope antibodies are examined.

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Identification and nucleotide sequence of a human locus homologous to the v-myc oncogene of avian myelocytomatosis virus MC29

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Avian myelocytomatosis virus MC29 is a replication-defective acute leukaemia virus which induces a variety of tumours in chickens including sarcomas, renal and hepatic carcinomas, and myelocytomatosis¹. The oncogenic potential of the virus is mediated by the gene v-myc, acquired from sequences (c-myc) present in normal uninfected chicken DNA². Sequences closely related to chicken c-myc have been highly conserved throughout evolution, from *Drosophila* to vertebrates^{3,4}. The hypothesis that c-myc may be involved in neoplastic transformation has been strengthened by the finding that B-cell lymphomas induced in chickens by avian leukosis virus (ALV) are often associated with increased expression of c-myc resulting from integration of the ALV provirus adjacent to the c-myc gene^{5,6}. More recently, it has been demonstrated that the malignant human cell line HL-60, derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukaemia, expresses elevated levels of myc-related mRNA⁷ associated with an amplification of the c-myc gene^{8,9}. To explore the relationship of the human cellular myc gene with the corresponding viral oncogene from MC29, and to provide a framework for the analysis of the mechanism and significance of c-myc amplification in human tumours, we have isolated and determined the nucleotide sequence of a genomic clone prepared from a normal human library which contains all domains sharing homology with v-myc.

A human recombinant DNA library¹⁰ was screened using a 1,519-base pair (bp) *Pst*I fragment from cloned MC29 DNA¹¹

which encompasses most of the myc-specific sequences of the virus^{11,12}. From approximately 600,000 phage plaques, we isolated six clones which displayed strong hybridization to the probe, four of which were unique as revealed by restriction enzyme mapping. The extent of v-myc-related sequences represented in each of the four clones was assessed by hybridizing various restriction enzyme digests of each phage DNA with a probe specific for either the 5' portion or the 3' portion of v-myc. One clone contained sequences homologous to only the 5' probe therefore was not characterized further: its properties, however, were suggestive of a myc pseudogene or a distantly related gene as has been described recently¹³. The remaining three clones hybridized strongly in stringent conditions to both 5' and 3' probes, suggesting that they contained sequences homologous to most of the c-myc gene. Figure 1a presents a restriction map of one such clone (λM1). With each clone, hybridization could be localized to a 4.5-kilobase (kb) fragment after digestion with *Xba*I and *Eco*RI. This fragment was therefore subcloned into a plasmid vector for further analysis, and mapped with additional restriction endonucleases. As determined by hybridization, there were two regions which were homologous to the viral gene, interrupted by a region of non-homology spanning ~1.4 kb. The order of the two domains with respect to the direction of v-myc gene transcription was determined using v-myc probes representing 5'- or 3'-terminal parts of the v-myc gene; this information is summarized in Fig. 1b.

Previous work has indicated that the human genome contains a single functional c-myc gene¹³. Our hybridization data indicated that λM1 contained sequences homologous to most or all of v-myc, and was otherwise extremely similar with regard to restriction endonuclease sites to a genomic clone thought to represent the human c-myc locus¹³. We therefore determined the nucleotide sequence of the relevant portion of λM1 by cloning suitable restriction fragments into M13 phage vectors mp8 and mp9 and sequencing the inserts by the chain terminator technique¹⁴. Figure 2 illustrates the sequence of ~4,000 nucleotides, together with the deduced amino acids they encode. Two regions containing extensive open reading frames are present, separated by 1,376 nucleotides. A comparison of the deduced

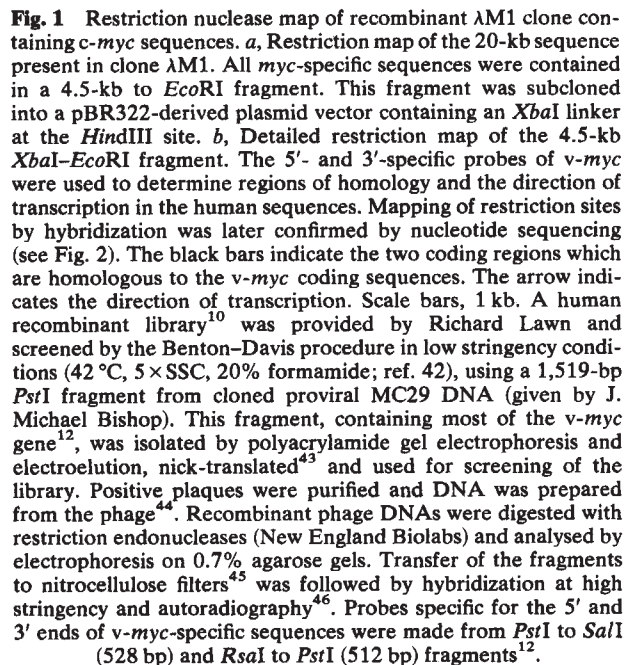
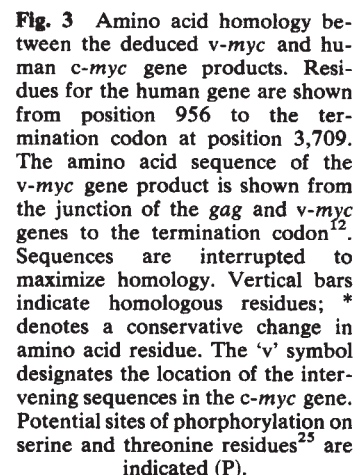
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Fig. 2 Nucleotide sequence of the human *c-myc* gene. The nucleotide sequence of 4,053 bp rightward of the *Xba*I site is presented (see Fig. 1). Restriction fragments were isolated as described in Fig. 1 legend and cloned into various sites of M13 phage vectors mp8 and mp9 (ref. 47). Sequencing was performed by the chain terminator technique¹⁴. The predicted amino acids are shown for the two domains of the open reading frame. A Goldberg-Hogness sequence (TATTTAT)^{29,30} at position 333, an RNA cap site (CCACTCC)³¹ at position 371, consensus splice sites (TCTG|GTAAAC) and (TTTCTTAAAG|AG)²⁰ at the junctions of the intervening sequences, and the hexanucleotide signal specifying polyadenylation (AATAAA)³⁴ at position 4,005, are underlined.



The presence of an in-frame translational termination codon in the human *c-myc* gene at a position identical to that in *v-myc* establishes the 3' boundary of the coding region of the human gene with some certainty (see Figs 2, 3). Inspection of the sequence reveals the hexanucleotide AATAAA which follows the translational termination codon by 300 nucleotides, thought to represent a signal for polyadenylation of mRNAs³⁴. If the interpretation of these features of the *c-myc* gene is correct, the estimated size of a mRNA transcript from the 5' end at position 371 to the site of polyadenylation at 4,038 would be ~2,300 bases, after removal of the 1,376-bp intron. When allowance is made for the addition of polyadenylate residues,

the length of the predicted transcript is in good agreement with experimentally determined size estimates of 2.2–2.7 kb (refs 7, 35, 36). However, this interpretation must be considered tentative, and again awaits detailed analysis of the corresponding mRNA for confirmation.

Transduction of cellular genes by retroviruses is thought to involve the capture of a cellular gene by the DNA provirus, facilitated perhaps by a further recombinational event during a subsequent round of viral infection^{18,37}. Although *v-myc* was derived from the *c-myc* gene of the chicken², a comparison of the *v-myc* sequence with that of human *c-myc* nonetheless provides some indication of the events which may have resulted in the generation of MC29. At the 5' end of the *v-myc* gene (where the sequence of MC29 diverges from that of Rous sarcoma virus, RSV¹²), significant homology is observed with the human cellular locus (Fig. 4) beginning just upstream of the methionine codon which may represent the translational initiation codon for human *c-myc*. If so, this suggests that one recombinational event leading to the capture of *c-myc* may have involved the juxtaposition of sequences within an exon just upstream from the translational initiation codon of *c-myc* and sequences in *gag* encoding p27 (ref. 12). This is in contrast to the findings with *c-myc*³⁸ where the leftward boundary of transduced cellular sequences lies within an intron. However, present models for transduction allow for either possibility at the leftward recombination junction^{18,19}. The homology between *v-myc* and *c-myc* continues (except for the single intron) past the translational termination codons near the 3' ends of the genes, ending at position 3,931 in *c-myc*, precisely where the *v-myc*-specific sequences end and are joined to the envelope gene in MC29 (ref. 12). Note that homology between *v-myc* and human *c-myc* terminates 74 nucleotides before the AATAAA hexanucleotide of the human gene: the absence of this putative polyadenylation signal from MC29 may ensure that transcription does not terminate before reaching the 3' long terminal repeat of the provirus, an important consideration in the retroviral life cycle¹⁸.

Although the precise role of the *c-myc* gene product in neoplastic transformation has not been elucidated, a correlation of the transformed phenotype with altered expression of the gene has been described in several systems^{5–7,35}. Whether increased expression alone is sufficient to effect transformation is not clear at present. The possibility exists that specific mutations in the normal cellular gene give rise to a mutated gene product with transforming potential; the recent demonstration that a single amino acid change can activate a normal human oncogene lends support to this mechanism^{39–41}. Detailed comparison of the *c-myc* gene or rearranged versions of it from appropriate human neoplastic cells with the normal gene described here should reveal the features accounting for the altered expression and may clarify the role of the *c-myc* gene in oncogenesis.

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An unusual mitochondrial DNA plasmid in the genus *Brassica*

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Mitochondria of flowering plants contain a diverse collection of small circular and linear plasmid-like molecules^{1–11} that replicate autonomously from the highly complex and variable chromosomal genome^{12,13}. Much interest and research has focused on two sequence elements, present in the mitochondrion as linear autonomous replicons in certain cytoplasmic male sterile (cms) lines of corn^{1–3} and sorghum^{8,9}, which in the case of corn are found to be integrated in the mitochondrial chromosome in normal and spontaneously revertant fertile lines^{14–16}. We describe here an extrachromosomal, plasmid-like DNA species found in the mitochondria of certain species of the genus *Brassica*. Its properties include the following: (1) the plasmid, found thus far only in the diploid species *Brassica campestris* and the allotetraploid *Brassica napus*, is phylogenetically distributed across a group of 20 *Brassica* cytoplasmic male sterile lines in a manner suggesting its loss or gain on multiple independent occasions. (2) The copy number of the plasmid varies ~100-fold in those accessions where it is found—from 1/10 to 10 times that of the main mitochondrial genome. (3) The plasmid is 11.3 kilobase pairs (kbp) in size, is linear in conformation, has no detectable internal repeats and is highly conserved in nucleotide sequence. (4) The presence and abundance of the plasmid show a strong association with cytoplasmic male sterility. (5) The plasmid shows no homology to chromosomal mitochondrial DNA from any of the *Brassica* accessions examined, nor is it homologous to the linear S₁ and S₂ mitochondrial plasmids found in S-cms cytoplasmic male sterile lines of corn.

Mitochondrial (mt) DNA was purified¹⁷ from 20 accessions (Table 1) of the seven economically important species of *Brassica* and radish (*Raphanus sativus*). All the mtDNAs contain a major component consisting of an unresolved smear of

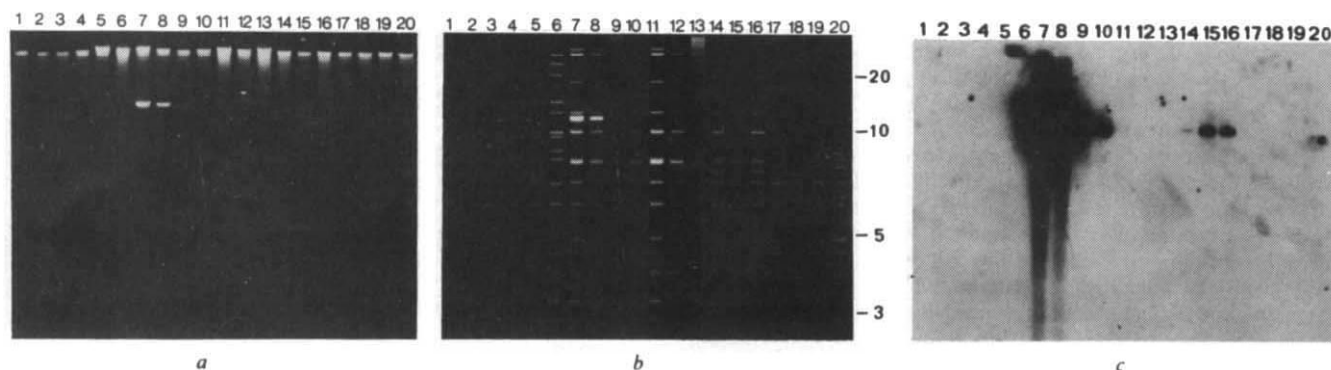


Fig. 1 Distribution of a mitochondrial DNA plasmid in the genus *Brassica*. *a*, Fractionation of DNase I-purified¹⁷ mitochondrial DNAs from samples 1–20 (Table 1) by electrophoresis on a 0.7% agarose gel²¹. *b*, Same as above except DNAs were digested with *SalI* before electrophoresis. *c*, Hybridization of ³²P-nick-translated²⁰ mitochondrial DNA plasmid purified¹⁹ by preparative gel electrophoresis from DNA 7 to a nitrocellulose filter blot²¹ of the gel shown in *b*. Vertical size scale is in kbp.

high molecular weight DNA which forms the main mitochondrial genome^{12,13} (Fig. 1*a*). In addition, mtDNAs of accessions 7 and 8, and more faintly, mtDNAs 9, 10, 15 and 16, contain a discrete molecular species with an electrophoretic mobility corresponding to that of a linear molecule 11.3 kbp in length (Fig. 1*a*). This molecule consistently co-purified with the main mitochondrial genome extracted from DNase I-treated organelles.

Digestion with the restriction endonuclease *SalI* converts the high molecular weight smear into a series of discrete bands without affecting the 11.3-kbp component (Fig. 1*b*). The *Brassica SalI* patterns give a genome size estimate of ~230 kbp and are among the simplest reported for a plant mitochondrial genome^{12,13}. Critical analysis of the differences between accessions with respect to the main mitochondrial genome requires a complete physical map of sites for *SalI* and other enzymes and will be published elsewhere.

These *SalI* patterns (Fig. 1*b*) generally resemble those reported by Lebacqz and Vedel¹⁸, with the exception that their pattern derived from *Brassica nigra* is quite different from ours, and is identical to both their and our *B. campestris* and *Brassica juncea* patterns. Given the strong differentiation of both the mitochondrial (Fig. 1*b*) and chloroplast (Fig. 2) genomes of *B. nigra* from those of *B. campestris* and *B. juncea*, we suggest that the *B. nigra* used by Lebacqz and Vedel¹⁸ was either misidentified or has cytoplasm derived from *B. campestris-juncea*.

To assay more sensitively for the presence of the plasmid and to examine possible plasmid homologies with chromosomal mitochondrial DNA, the 11.3-kbp extrachromosomal band was purified¹⁹ from mtDNA 7, labelled with ³²P by nick-translation²⁰ and used as a hybridization probe against a nitrocellulose filter imprint²¹ of the gel shown in Fig. 1*b*. Whereas the 11.3-kbp element was not detectable on ethidium bromide staining of mtDNA 14 (Fig. 1*a, b*), the more sensitive hybridization assay reveals the plasmid molecule, at a copy number estimated to be only 1/10th that of the main genome (Fig. 1*c*). Fivefold longer autoradiographic exposure fails to reveal the plasmid in any DNAs other than those of accessions 7–10 and 14–16, indicating that if the plasmid is present in these DNAs it is at stoichiometric levels considerably below 1/50th that of the main genome. Note that the copy number variation shown in Fig. 1 is highly reproducible. Examination of total cellular DNA prepared from each of five individual plants from accessions 3, 6–11, 14–16, 19 and 21–24 failed to reveal any plant-to-plant variation in plasmid levels (normalized relative to the main mitochondrial genome) within an accession, while relative plasmid levels between accessions were equivalent to those shown in Figs 1 and 4 (data not shown).

No detectable homology was observed between the plasmid and chromosomal mtDNA *SalI* fragments, even at autoradiographic exposures five times beyond that shown in Fig. 1*c*. This result contrasts with the observation that sequences

homologous to the S₁ and S₂ plasmid-like DNAs found in mitochondria of the S-cms cytoplasm of corn are present in the main mitochondrial genome of fertile corn lines^{14–16}.

The observed phylogenetic distribution of the mitochondrial plasmid only sporadically corresponds to a chloroplast DNA-based *Brassica* phylogeny (Fig. 2). The three most parsimonious explanations which could account for these incongruities in plasmid distribution all postulate at least three independent losses or gains of the plasmid. The three explanations postulate: (1) a derived gain in accessions 1–3 and 7–16 and independently derived losses in 1–3 and in 11–13; (2) a gain in 14 and 16, a gain in 7–13 and 15, and a loss in 11–13; (3) a gain in 9, a gain in 14 and 16, and a gain in 7, 8, 10 and 15. Given these results, indicating that evolutionary loss or gain of the plasmid may be fairly common events, caution should be exercised in drawing any broad conclusions about the presence or absence of the plasmid in a particular *Brassica* species. The failure to find the plasmid in one or more accessions of a given species does not mean that other accessions of the same species will not be found to contain the plasmid; as indicated below, this issue is most crucial with respect to radish cytoplasms and their involvement in cytoplasmic male sterility.

The physical structure of the 11.3-kbp element was investigated by restriction fragment analysis and mapping. The sum of fragments generated by single digestion with 29 different enzymes and also by various double digestions always equalled ~11.3 kbp. However, the number of fragments produced in double digestions was always one less than the sum number of fragments produced by single digestions with the same two enzymes. This suggests that all enzymes share a common site, a result compatible with the hypothesis that the plasmid is a linear molecule with unique ends. Thirty sites, produced by 13 different restriction enzymes, were mapped on the linear 11.3-kbp molecule by appropriate double digestions of total mtDNA (Fig. 3). No internal duplications are apparent from the map and thus any internal homologies present must be relatively short, no more than a few hundred base pairs in length. The S₁ and S₂ plasmid molecules found in mitochondria from S-cms corn possess terminal inverted repeat sequences of 0.2 kbp²². Our mapping data suggest an upper limit of 0.5 kbp for any analogous sequences in the *Brassica* plasmid. Electron microscopic analysis of self-renatured molecules and cross-hybridization between subfragments of the molecule are now in progress to resolve whether this plasmid contains any internal homologies.

We have examined the relationship between the mitochondrial plasmid and two cms systems in *Brassica*. As a result of tissue limitations, mitochondrial DNA from cms *B. campestris* and *B. napus*, as well as from fertile, isonuclear maintainer lines, was prepared from crudely purified mitochondria²¹ and thus may contain considerable amounts of contaminating chloroplast and nuclear DNA. In spite of this limitation, agarose gel electrophoresis clearly shows that cms *B. campestris* contains

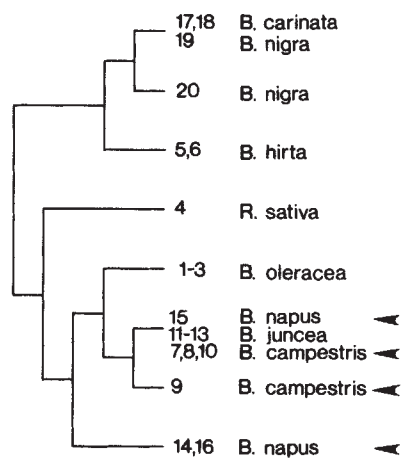


Fig. 2 Maternal *Brassica* phylogeny based on chloroplast DNA restriction site mutations²⁵. Arrows denote those accessions (numbered according to Table 1) which contain the 11.3-kbp mitochondrial plasmid (Fig. 1).

high levels of the plasmid (Fig. 4, lanes 2, 5). The plasmid copy number is estimated to be 10-fold higher than that of the main mitochondrial genome based on hybridization with either total mtDNA or cloned mtDNA restriction fragments (data not shown). In contrast, hybridization analysis indicates that the plasmid is greatly reduced in abundance in a fertile isonuclear maintainer line which has *B. campestris* cytoplasm (Fig. 4, lane 10; a low level of plasmid in the maintainer line was observed in several different hybridizations, including one where empty gel lanes bracketed the maintainer DNA, and thus the hybridization shown in lane 10 does not result from spillover of DNA from lane 9).

This cms *B. campestris* line was developed by nuclear substitution (of the fertile maintainer *B. campestris* line) into cms *R. sativus* cytoplasm via backcrossing²³. Electrophoretic analysis of ctDNA and mtDNA restriction patterns from these accessions confirms that this cms *B. campestris* does indeed carry radish cytoplasm, that is, the main mitochondrial and chloroplast genomes are, as expected, inherited maternally in

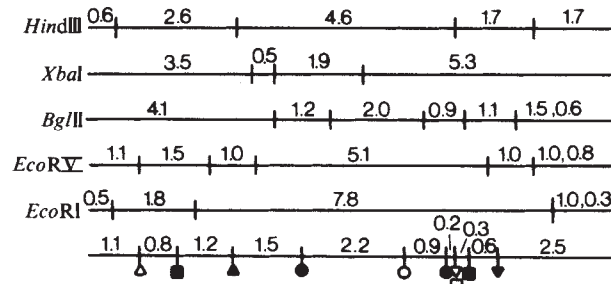


Fig. 3 Restriction map of linear *Brassica* mitochondrial DNA plasmid. Total mitochondrial DNA from sample 7 was treated with 29 different restriction endonucleases. 13 of the enzymes (*BalI*, *BglI*, *KpnI*, *MstII*, *NcoI*, *NruI*, *PstI*, *PvuI*, *SacII*, *SalI*, *SmaI*, *StuI* and *TthI*) did not cut the plasmid. Sites of all but three (*BclI*, *EcoRII* and *XmnI*) of the enzymes which did cut the plasmid were mapped by various double digestions of total mitochondrial DNA, followed by transfer of fragments to nitrocellulose filters and hybridization with ³²P-nick-translated²⁰ mitochondrial DNA plasmid purified¹⁹ by preparative gel electrophoresis. Fragment sizes are in kbp. Restriction sites shown: ○, *BamHI*; ●, *BstEII*; ●, *CfoI*; ●, *HpaI*; △, *PvuII*; ◆, *SacI*; ▲, *SphI*; □, *XhoI*.

Brassica and *Raphanus* (data not shown). Because the single fertile *R. sativus* line examined in this study does not contain the plasmid (Fig. 1c), the existence of the plasmid in great abundance in this cms line raises intriguing questions concerning a possible non-maternal, even nuclear, origin and mode of transmission for the plasmid. Clearly, a variety of biochemical and genetic approaches—survey of additional fertile and cms radish cytoplasm for the plasmid, examination of the nuclear genome for possible homologues to the mitochondrial plasmid, analysis of the reciprocal introgression of the introgressed radish cytoplasm back into a radish nuclear background—are needed to test these possibilities.

The mitochondrial plasmid also seems to be associated with cms in a cms *B. napus* line which has *B. napus* cytoplasm (judged by restriction analysis of its ctDNA and mtDNA, data not shown). The plasmid is present in moderate abundance in the cms line (Fig. 4, lane 11), but is not detected in the fertile, isonuclear maintainer line (Fig. 4, lane 12). These results contrast with those obtained by Vedel *et al.*²⁴ in their examination

Table 1 Source of *Brassica* DNAs

	Species	Origin	Line information	Plasmid*
(1)	<i>Brassica oleracea</i>	Europe	cv. Cruiser (broccoli)	—
(2)	<i>Brassica oleracea</i>	Europe	cv. Snowball (cauliflower)	—
(3)	<i>Brassica oleracea</i>	Europe	cv. Oscar (cabbage)	—
(4)	<i>Raphanus sativus</i>	Korea	Plant Introduction (sps)	—
(5)	<i>Brassica hirta</i>	Europe	Commercial white mustard line	—
(6)	<i>Brassica hirta</i>	Ethiopia	Plant Introduction 195, 922 (sps)	—
(7)	<i>Brassica campestris</i>	Canada	cv. Torch (oil seed)	+
(8)	<i>Brassica campestris</i>	California	Volunteer weed population (sps)	+
(9)	<i>Brassica campestris</i>	India	Subspecies <i>trilocularis</i> PI 180, 412 (sps)	+
(10)	<i>Brassica campestris</i>	China	Plant Introduction	+
(11)	<i>Brassica juncea</i>	Canada	cv. Domo (oil seed)	—
(12)	<i>Brassica juncea</i>	India	Plant Introduction NU60039 (sps)	—
(13)	<i>Brassica juncea</i>	China	Plant Introduction (sps)	—
(14)	<i>Brassica napus</i>	Canada	cv. Altex (oil seed)	+
(15)	<i>Brassica napus</i>	Poland	cv. Bronowski	+
(16)	<i>Brassica napus</i>	Japan	cv. Norin 31 (sps)	+
(17)	<i>Brassica carinata</i>	India	Plant Introduction 181, 033 (sps)	—
(18)	<i>Brassica carinata</i>	Ethiopia	Plant Introduction 193, 759 (sps)	—
(19)	<i>Brassica nigra</i>	India	Plant Introduction 179, 860 (sps)	—
(20)	<i>Brassica nigra</i>	Europe	Commercial black mustard line	—
(21)	<i>Brassica campestris</i>	USA	Male-sterile (radish cytoplasm)	+
(22)	<i>Brassica campestris</i>	USA	Fertile (maintainer line for 21)	+
(23)	<i>Brassica napus</i>	Korea	Male-sterile (<i>B. napus</i> cytoplasm)	+
(24)	<i>Brassica napus</i>	Korea	Fertile (maintainer line for 23)	—

sps, Seed for plants used was derived from a single plant selection.

* Plus indicates the presence and minus indicates the absence of the 11.3-kbp linear molecule in mitochondrial DNA prepared from each of the accessions (see Figs 1 and 4).

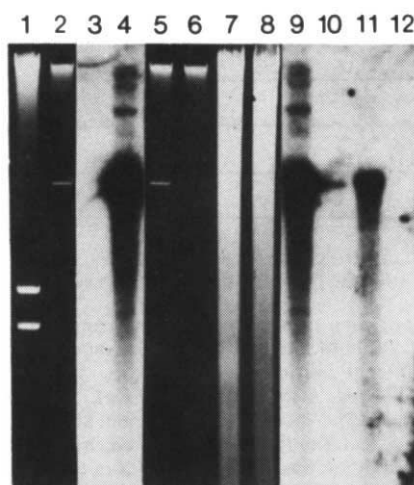


Fig. 4 Lack of homology of *Brassica* mitochondrial plasmid to S_1 and S_2 plasmid DNAs from corn mitochondria and association of the *Brassica* plasmid with cytoplasmic male sterility. DNase I-purified¹⁷ mitochondrial DNA from *S* cytoplasm of corn (lane 1) and sucrose gradient-purified²¹ mitochondrial DNA from accessions 21 (lanes 2, 5) and 22–24 (lanes 6–8, respectively) were fractionated by electrophoresis on a 0.7% agarose gel²¹, transferred to nitrocellulose filter paper²¹ and hybridized²¹ with ³²P-nick-translated²⁰ mitochondrial DNA plasmid purified¹⁹ by preparative gel electrophoresis from DNA 7. Lanes 3 and 4 are autoradiographs of the hybridization to nitrocellulose blots of the gel lanes shown in lanes 1 and 2, while lanes 9–12 are autoradiographs corresponding to the gel lanes in lanes 5–8. Filters were washed at 60 °C in 2×SSC, 0.1% SDS before autoradiography. 0.5 µg DNA was loaded in lanes 2, 5 and 6, 3 µg in lane 1 and 20 µg in lanes 7 and 8.

of *B. napus* cms and maintainer lines. Their cms *B. napus* derived its cytoplasm from the same cms radish line as our cms *B. campestris*. However, the plasmid was not detected in either their cms or fertile lines, although its presence cannot be ruled out on the basis of visual inspection of agarose gels alone²⁴ (see Fig. 1).

The mitochondrial plasmids present in fertile *Brassica* lines 7–10, 15 and 16 and cms lines 21 and 23 (Table 1) are identical at all 41 sites, representing 240 nucleotide pairs, sampled with 8 different restriction enzymes (data not shown). Chloroplast DNAs from *B. campestris* and radish differ by 1.1% in nucleotide sequence²⁵ and preliminary analysis suggests that their mitochondrial genomes are significantly more divergent (see, for example, Fig. 1b). Hence, if the mitochondrial plasmid in the cms *B. campestris* was derived from the radish cytoplasm, it must have evolved quite slowly relative to both the main mitochondrial genome and the highly conserved chloroplast genome. This sequence identity between the mitochondrial plasmids of fertile and cms *B. campestris* suggests a very recent common origin—through either a shared *B. campestris* cytoplasm or nucleus.

Although the *Brassica* mitochondrial plasmid resembles the corn S_1 and S_2 plasmid molecules^{1–3} in its linearity and association with cms, it shows no sequence homologies to these molecules (Fig. 4, lane 3). Furthermore, no homologies were observed in reciprocal hybridizations between the *Brassica* mitochondrial plasmid and cauliflower mosaic viral DNA (data not shown).

The strongest association between the 11.3-kbp linear molecule and the mitochondrion is physical—the plasmid consistently co-purified with the main mitochondrial genome during subcellular fractionation. In contrast, little or no association was found between the two genomes in several other traits—the plasmid shows no sequence homology to the mitochondrial genome, is distributed sporadically on a cytoplasmic phylogenetic tree, seems to be evolving more slowly than mitochondrial DNA, and may even be transmitted independently of the

mitochondrial genome. As discussed earlier, this last observation, together with the whole question of the precise involvement of the plasmid in cytoplasmic male sterility, deserves intensive biochemical and genetic examination. Moreover, linear molecules often use quite bizarre means to ensure their replication, and the 100-fold natural variation in plasmid copy number suggests its use as an excellent model system in which to study the regulation of extrachromosomal DNA replication. The question of plasmid expression clearly needs to be addressed—as a basic question in its own right, in relation to its role in cms, and as a model system for exploring gene dosage effects on gene expression.

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***Klebsiella pneumoniae nifA* product activates the *Rhizobium meliloti* nitrogenase promoter**

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Bacteria in the genus *Rhizobium* normally fix nitrogen only when they interact with leguminous plants to produce on the roots a highly differentiated structure, the nodule, within which the bacteria differentiate into nitrogen-fixing bacteroids. By contrast, the enteric bacterium *Klebsiella pneumoniae* reduces nitrogen in a free-living state in conditions of low oxygen tension and deficiency of fixed nitrogen. In *K. pneumoniae*, the overall circuitry by which nitrogen-fixation (*nif*) genes are regulated has been elucidated^{1–4}. In response to ammonia starvation, the product of the *glnG* gene activates transcription of the *nifLA*

operon; this activation is dependent on the product of *glnF* (ref. 4). The *nifA* gene product is in turn required for transcription of all the other *nif* genes, including the *nifHDK* operon which codes for the subunits of nitrogenase. In contrast, very little is known about the sequence of events involved in the regulated change in rhizobial *nif* gene expression associated with bacteroid differentiation. In the work described here, we identify the *K. pneumoniae* and *Rhizobium meliloti* *nifHDK* promoters by mapping the *in vivo* start points of transcription. By defining and comparing the DNA sequences of these two promoters, we find that they share an unexpected degree of homology. Further, by constructing fusions of each of the two promoters to the *lacZ* gene from *Escherichia coli*, we show that both promoters are activated by the product of the *K. pneumoniae* *nifA* gene.

We have described previously the cloning and preliminary genetic characterization of the *R. meliloti* *nifHDK* operon⁵⁻⁷. Because the amino acid sequence of the *nifH* gene has been highly conserved between *K. pneumoniae* and *R. meliloti*, we can deduce, by DNA sequence analysis (not shown), that the translational start point of the *nifH* gene in *R. meliloti* lies within a 710-base pair (bp) *Xho*I fragment (refs 7, 8 and Fig. 1a). This fragment was used to probe RNA isolated from bacteroids as well as from free-living *R. meliloti* by hybridization followed by treatment with *S*₁ nuclease^{9,10}. A *nif*-specific transcript starting at about 290 nucleotides from the *Xho*I site was detected in the bacteroid RNA, but not in the RNA from the free-living cells (Fig. 2a). The exact start point of transcription was determined by electrophoresis of the *S*₁-protected DNA adjacent to a DNA sequencing ladder; it was found to be 69 nucleotides upstream from the ATG codon. We determined the DNA sequence of the entire *Xho*I fragment; the promoter-proximal region, including -10 and -35 sequences, is shown in Fig. 3. This is the first determination of a symbiotically active (that is, bacteroid) promoter at the level of DNA sequence.

To determine the transcription start site of the *K. pneumoniae* *nifH* gene, we followed the strategy described above, using as probe a 396-bp *Eco*RI-*Sau*3A fragment spanning the ATG initiation codon of *K. pneumoniae* *nifH* (ref. 11 and Fig. 1b). RNA was isolated from cultures that were grown with NH₄⁺ (repressed) and without NH₄⁺ (derepressed). A transcript was detected only in the derepressed cells (Fig. 2b); the start of transcription was 30 nucleotides upstream from the ATG start codon (Fig. 3).

The DNA sequences of both *nifH* promoters are shown in Fig. 3. The sequences at -35 and -10 are important for recognition by prokaryotic RNA polymerases^{12,13}. The consensus sequences (based on data for *E. coli* RNA polymerase) are TTGACA at -35 and TATAAT at -10 (ref. 12). The same sequences are recognized by other Gram-negative RNA polymerases¹⁴, including the RNA polymerase from *Rhizobium leguminosarum*, the nitrogen-fixing endosymbiont of pea¹⁵. Neither the *K. pneumoniae* nor the *R. meliloti* *nifH* promoter exhibits good homology at -10 and -35 to these consensus sequences. This observation is consistent with our inability to detect significant transcription from either of the two promoters *in vitro*, using *E. coli* RNA polymerase and purified DNA fragments as templates (Fig. 2a, lane 5 and Fig. 2b, lane 6). The above data support the idea that the *K. pneumoniae* and *R. meliloti* *nifHDK* promoters require activator proteins for transcription.

Although the DNA sequences of the *K. pneumoniae* and *R. meliloti* *nifHDK* promoters show poor homology to consensus sequences, they show surprisingly good homology to each other. There is ~50% homology between the promoters for the region between -40 and +1, and there are regions of exact homology at -70 (6 bp), -32 (8 bp) and -14 (5 bp), as shown in Fig. 3. [The nucleotide numbers refer to the *R. meliloti* *nifH* promoter; they differ from the *K. pneumoniae* promoter by 1-2 nucleotide positions.] The conservation of an 8-nucleotide sequence, ACGGCTGG, starting at -32, is particularly inter-

esting, as this region of certain promoters has been found to be involved in recognition by activator proteins^{12,16}.

The *K. pneumoniae* *nifH* promoter is activated by the product of the *K. pneumoniae* *nifA* gene³. The DNA sequence homology that we found between the *R. meliloti* and *K. pneumoniae* *nifHDK* promoters raised the intriguing possibility that the *K. pneumoniae* *nifA* gene product might be able to activate the *R. meliloti* *nifH* promoter. To investigate this possibility, we designed an experiment in which we could readily monitor the activity of the *R. meliloti* and *K. pneumoniae* promoters in an *E. coli* strain which constitutively produced the *K. pneumoniae* *nifA* gene product. The activity of the *nifHDK* promoters was monitored by constructing translational *nifH*:*lacZ* fusions using *lacZ* from *E. coli*, as shown in Fig. 1. Plasmid pVSA2 carries a fusion of the first codon of *K. pneumoniae* *nifH* to the 8th codon of *lacZ* (Fig. 1b), and plasmid pVSP9 carries a fusion of the 29th codon of *R. meliloti* *nifH* to the 8th codon of *lacZ* (Fig. 1a). These fusions are carried on cloning vectors derived from pBR322¹⁷. The *nifA* gene product was produced in *E. coli* by means of plasmid pDO202, which contains a transcriptional fusion of the *lacZ* promoter (*P*_{lacUV5}) to *K. pneumoniae* *nifA* on a phage P4 vector that is compatible with pBR322. The experiment was designed to introduce pDO202 into a Δ(*lacIZY*) *E. coli* strain containing either pVSA2 or pVSP9. An increase in the level of β-galactosidase would indicate that the *nifA* gene product activates the *nifHDK* promoters. Because it has recently been observed that *nifA* and *glnG* can sometimes recognize and activate the same promoters⁴, the *E. coli* strain we used (YMC11) contained a deletion of the *glnALG* operon; thus, any activation detected could only be due to *nifA*.

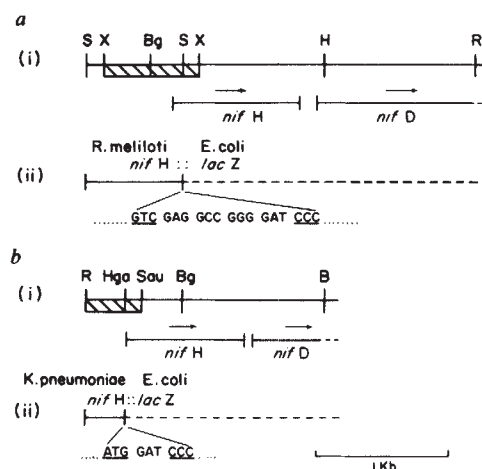


Fig. 1 Restriction maps of the *nifHDK* promoter regions from *R. meliloti* and *K. pneumoniae* and structures of the *nifH*:*lacZ* fusions. *a*(i), *R. meliloti* *nifHDK* promoter. The arrows indicate direction of transcription and the boxed region shows the 0.71-kilobase (kb) *Xho*I fragment used as a probe in the *S*₁ mapping experiment described in the text. *a*(ii), *R. meliloti* *nifH*:*lacZ* fusion (on plasmid pVSP9). The 0.72-kb *Sal*I fragment containing the promoter was cloned into the *Bam*HI site of the *lacZ* containing plasmid pMC1403¹⁷ using *Xho*I linkers. The sequence at the junction is shown, where the 29th codon of *nifH* (GTC) has been joined to the 8th codon of *lacZ* (CCC). *b*(i), *K. pneumoniae* *nifHDK* promoter. The arrows indicate direction of transcription and the boxed region shows the 0.396-kb *Eco*RI-*Sau*3A fragment used as a probe in the *S*₁ mapping experiment (see text). *b*(ii), *K. pneumoniae* *nifH*:*lacZ* fusion (on plasmid pVSA2). The 0.291 kb *Eco*RI-*Hga*I fragment¹¹ was cloned into the *Bam*HI site of pMC1403¹⁷ by blunt-end ligation following filling-in of the protruding ends using DNA polymerase I (Klenow fragment). The sequence at the junction is shown, where the first codon of *nifH* (ATG) has been joined to the 8th codon of *lacZ* (CCC). Abbreviations: S, *Sal*I; X, *Xho*I; B, *Bgl*II; H, *Hind*III; R, *Eco*RI; Hga, *Hga*I; Sau, *Sau*3A. Details of the construction of pVSP9 and pVSA2 will be reported elsewhere.

Table 1 shows that the *K. pneumoniae* *nifHDK* promoter was activated by *nifA*, in agreement with earlier results⁵. We also found that the *R. meliloti* *nifH* promoter was activated by *nifA* (Table 1); the level of β -galactosidase measured was about half that obtained with *K. pneumoniae* *nifH*. In both cases, the activation did not significantly depend on NH_4^+ concentration. To rule out the possibility that a promoter in the vector (pBR322) was influencing the result, the *nifHDK-lacZ* promoter fusions were recloned into a phage P4 vector and the experiments repeated using the *lacUV5* promoter-*nifA* fusion recloned into pBR322 as the source of *nifA*. Once again, we observed that *nifA* activated both promoters (Table 1). In the latter case, there was a slightly higher basal (that is, without *nifA*) level of β -galactosidase with the *R. meliloti* promoter (relative to the *K. pneumoniae* promoter), probably due to readthrough from a phage P4 promoter. The cloned *K. pneumoniae* promoter fragment has a putative terminator sequence upstream from *nifH*¹⁸, and therefore might be protected from such readthrough.

We have thus shown that the *nifHDK* promoters from *K. pneumoniae* and *R. meliloti* share significant sequence

homology and that both promoters are activated by the *nifA* gene product of *K. pneumoniae*. In addition to the results reported here, the *R. meliloti* and *K. pneumoniae* *nifHDK* promoters have another feature in common. *K. pneumoniae* *nif* promoters cloned into multicopy plasmids inhibit nitrogen fixation in wild-type *K. pneumoniae* cells, presumably due to titration of *nifA* product¹⁹⁻²¹. The multicopy plasmid, pRmR2, containing the *R. meliloti* *nifH* promoter⁶, also inhibits *K. pneumoniae* nitrogen fixation by over 90%, suggesting that *R. meliloti* *nifH* promoter also binds *nifA* (V. Buchanan-Wollaston and F. C. Cannon, personal communication; V.S. and F.M.A., unpublished). We do not know if the sequence recognized by *nifA* is one of the conserved sequences shown in Fig. 3.

At least two of the nitrogenase genes of *K. pneumoniae* and *R. meliloti* (*nifH* and *nifD*) show considerable homology at both the DNA sequence and amino acid sequence levels^{5,7,8}. In both species, the *nifH*, *D* and *K* genes are arranged in the same order in a single operon, *nifHDK*⁷ (see ref. 22 for a recent review). The data presented here point to the existence of a 'N' protein in *R. meliloti*, suggesting that the *nif* genes along

Fig. 2 Mapping of *nifHDK* promoters using S_1 nuclease. **a**, S_1 mapping of *R. meliloti* *nifH* promoter. Lane 1: the 0.71-kb *XhoI* fragment used as the probe. Lanes 2-5: the S_1 protected fragments obtained by hybridizing RNA from different sources. Lanes 2, 3: two preparations of RNA from bacteroids. Lane 4: RNA from free-living *R. meliloti*. Lane 5: RNA obtained by the *in vitro* transcription of 0.2 pmol of the 0.71-kb *XhoI* fragment, using *E. coli* RNA polymerase at 10:1 excess in standard conditions²⁷. Some weak transcription can be observed in this latter experiment. The arrow indicates an *in vitro* transcript that has the same start point as the *in vivo* bacteroid transcript (determined by electrophoresis of the two samples on sequencing gels). However, as we used conditions of excess RNA polymerase, causing initiation at nonphysiological sites (that is, within the coding region), this transcript may not be physiologically significant. **b**, S_1 mapping of *K. pneumoniae* *nifH* promoter. Lane 1: molecular weight markers, including the 0.39-kb *EcoRI-Sau3A* fragment used as the probe. Lanes 2-6: the S_1 protected fragments obtained by hybridizing RNA from different sources. Lane 2: RNA from derepressed cells of KP5614. Lane 3: RNA from NH_4^+ repressed cells of KP5614. Lanes 4, 5: RNA from derepressed and NH_4^+ repressed cultures, respectively, of KP5614 containing the *nifHD* plasmid pSB1 (ref. 11). Lane 6: RNA obtained by the *in vitro* transcription of 0.2 pmol of the 0.73-kb *EcoRI-BglII* fragment containing the *nifH* promoter (Fig. 1), using the same conditions as before. No transcription could be detected. In the same conditions, the P-left promoter of phage λ was actively transcribed (not shown).

Methods: The DNA fragments used as probes are described in Fig. 1 and in the text. The probes labelled at the 5' ends with T4 polynucleotide kinase and the noncoding strands were isolated by the use of strand-separation gels¹⁰. The isolated single-stranded fragments were hybridized to RNA (isolated from bacteria or bacteroids as described below), and digested with S_1 nuclease. The S_1 -resistant DNA was electrophoresed on 8 M urea, 6% 1:30 polyacrylamide gels and autoradiographed. The procedures followed were those of Brosius *et al.*¹⁰ which are modifications of previously published methods^{8,28}. **Isolation of RNA from free-living *R. meliloti* strain 1021:** Cells from a 100-ml early log phase culture of *R. meliloti* in TY (0.5% tryptone, 0.3% yeast extract) streptomycin medium were spun down and resuspended in 1 ml of sterile 25% sucrose. This solution was transferred to a 30-ml Corex tube and 6.5 ml of M-STET (4% sucrose, 6% Triton X-100, 60 mM Tris-Cl pH 8.0, and 60 mM sodium EDTA pH 8.0), 0.5 ml of 10 mg ml⁻¹ lysozyme (freshly made up in sterile distilled water) and 0.4 ml of 200 mM vanadyl nucleoside RNase inhibitor²⁹ were added. The mixture was incubated on ice for 5 min. The Corex tube was placed in a boiling water bath for 2 min and spun at 8,000 r.p.m. in a Sorvall HB4 rotor for 10 min. The supernatant was precipitated with 0.7 volumes of isopropanol, pelleted in a Corex tube and redissolved in 5 ml of 100 mM LiCl, 1% SDS, 7 M urea, 100 mM Tris pH 8.4 and 5 mM EDTA (solution A). This solution was extracted three times with phenol/chloroform and precipitated with 2.5 vol of ethanol. The precipitate was redissolved in 2 ml of water which had been incubated with 0.1% diethylpyrocarbonate (DEP) for 2 h before autocleaving. 6 ml of DEP-treated 4 M sodium acetate pH 6.0 were added, the tube was incubated on ice for at least 2 h, and the precipitated RNA was spun out at 8,000 r.p.m. for 30 min and rinsed three times with cold DEP-treated 3 M sodium acetate pH 6.0. The RNA was redissolved in DEP-treated water, precipitated with ethanol and stored as an ethanol precipitate. RNA from bacteroids was isolated as follows: Alfalfa plants (variety Iroquois) were grown in a hydroponics apparatus on large trays. They were inoculated about 5 days after germination with *R. meliloti* strain 1021 and were ready for harvesting in 3-6 weeks. Nodules were hand-picked into vials in liquid nitrogen. 3 g of nodules were ground to a fine powder in a mortar and pestle containing liquid nitrogen. The powder was added to 5 ml of ice-cold 0.5 M mannitol, 50 mM Tris 7.5, 20 mM sodium succinate and 5 mM sodium dithionite. On resuspension, this material was passed through one layer of Miracloth and the Miracloth was rinsed with 5 ml of the same solution. The filtrate was collected in a 15-ml Corex tube and spun at 1,500 r.p.m. for 5 min at 4 °C in an HB4 rotor. The supernatant from this was spun at 9,000 r.p.m. for 5 min as above and the precipitate was brought up in solution A containing 10 mM vanadyl-ribonucleoside complex and treated in the same way as the first isopropanol precipitate in the preparation of free-living bacterial RNA described above. **Isolation of RNA from *K. pneumoniae*:** 5-ml cultures of *K. pneumoniae* strain KP5614 (*recA*⁻*hisD*⁻; ref. 21) were grown anaerobically to saturation at 30 °C in *nif*-derepression medium²³ supplemented with 0.2% (NH₄)₂SO₄ and 20 $\mu\text{g ml}^{-1}$ L-histidine, and then spun down and resuspended in *nif*-derepression medium with 20 $\mu\text{g ml}^{-1}$ histidine or in the same medium containing 0.2% (NH₄)₂SO₄ (which suppresses *nif* mRNA synthesis). The cells were incubated anaerobically for 6 h at 30 °C to allow derepression and accumulation of *nif* mRNA²¹. They were then centrifuged and lysed by essentially the same procedure as used for free-living *R. meliloti*. Following the clearing spin, the supernatant was precipitated with ethanol, resuspended in 0.3 M sodium acetate, extracted with phenol and chloroform, ethanol precipitated again, washed with 70% ethanol and dried down.

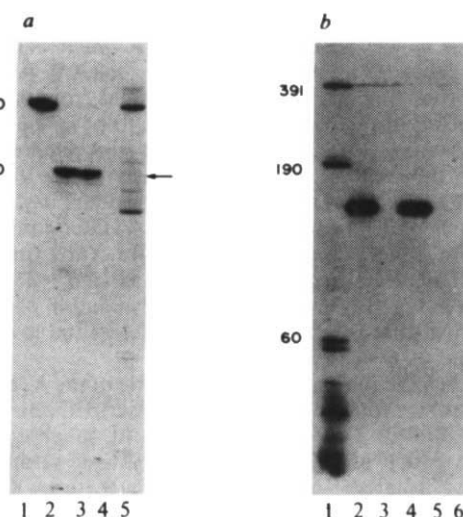


Table 1 Activation of the *K. pneumoniae* and *R. meliloti* *nifHDK* promoters by the *K. pneumoniae* *nifA* gene product

Strain	Relevant plasmid properties	β -Galactosidase units	
		$-\text{NH}_4^+$	$+\text{NH}_4^+$
YMC11 (pDO201) (pVSA2)	(Km^r , <i>nifLA</i> without P_{lacUV5}) (Ap^r , <i>K. pneumoniae nifH::lacZ</i>)	58	73
YMC11 (pDO202) (pVSA2)	(Km^r , $P_{\text{lacUV5}}::\text{nifA}$) (Ap^r , <i>K. pneumoniae nifH::lacZ</i>)	4,226	3,306
YMC11 (pDO201) (pVSP9)	(Km^r , <i>nifLA</i> without P_{lacUV5}) (Ap^r , <i>R. meliloti nifH::lacZ</i>)	54	49
YMC11 (pDO202) (pVSP9)	(Km^r , $P_{\text{lacUV5}}::\text{nifA}$) (Ap^r , <i>R. meliloti, nifH::lacZ</i>)	1,721	1,968
YMC11 (pBR322) (pVSA3)	(Ap^r) (Km^r , <i>K. pneumoniae nifH::lacZ</i>)	15	12
YMC11 (pDO516) (pVSA3)	(Ap^r , $P_{\text{lacUV5}}::\text{nifA}$) (Km^r , <i>K. pneumoniae nifH::lacZ</i>)	3,355	3,903
YMC11 (pBR322) (pVSP9-1)	(Ap^r) (Km^r , <i>R. meliloti nifH::lacZ</i>)	125	92
YMC11 (pDO516) (pVSP9-1)	(Ap^r , $P_{\text{lacUV5}}::\text{nifA}$) (Km^r , <i>R. meliloti nifH::lacZ</i>)	2,312	1,579

Cultures (5 ml) of the strains were grown to saturation at 30 °C anaerobically in *nif*-derepression medium²³ with 0.2% (NH_4)₂SO₄ supplemented with 0.2% L-glutamine, 10 $\mu\text{g ml}^{-1}$ kanamycin (Km) and 50 $\mu\text{g ml}^{-1}$ ampicillin (Ap), and resuspended in *nif*-derepression medium with or without 0.2% (NH_4)₂SO₄ (as indicated) and containing antibiotics as above, but only 100 $\mu\text{g ml}^{-1}$ of L-glutamine. The cultures were incubated for 10 h anaerobically at 30 °C, centrifuged and the β -galactosidase activity was measured as described by Miller²⁴. YMC11 is an *E. coli* K-12 strain; the relevant genotype is *supE44Δ(lacIPOZY)U169Δ(glnA-glnG)2000*²⁵. Plasmids pVSA2 and pVSP9 carry, respectively, the *K. pneumoniae* and *R. meliloti nifH::lacZ* fusions described in the text; both plasmids confer Ap resistance. Plasmids pVSA3 and pVSP9-1 are Km^r plasmids constructed by recloning the *nifH::lacZ* fusions from pVSA2 and pVSP9, respectively, into a phage P4 vector²⁶. The Ap^r plasmid pDO516 has been described elsewhere⁴; it carries the $P_{\text{lacUV5}}::\text{nifA}$ fusion. The cloning vector is pBR322. Plasmid pDO202 carries the same $P_{\text{lacUV5}}::\text{nifA}$ fusion on a Km^r phage P4 vector, and plasmid pDO201 carries the *nifLA* region, without the *lac* promoter, on the same P4 vector. Details of the construction of these plasmids will be described elsewhere.

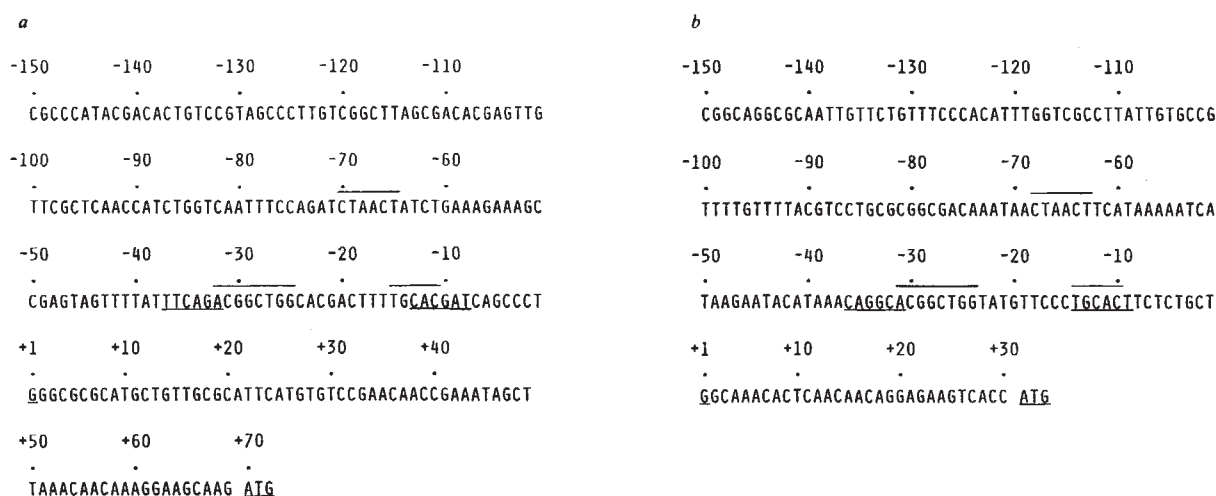


Fig. 3 Promoter sequences of *NifHDK* operons from *R. meliloti* (a) and *K. pneumoniae* (b). Start points of transcription were established by electrophoresis of the S_1 -protected fragments shown in Fig. 2 adjacent to a 'Maxam-Gilbert' sequencing ladder derived from the same probe^{10,28}. These determinations are usually accurate to ± 1 nucleotides for prokaryotic (uncapped) mRNAs (ref. 10). The -35 and -10 sequences are underlined and regions of exact homology are overscored. The region downstream from -70 has been sequenced in another strain of *R. meliloti*, although the location of the promoter was not determined⁴; there are only a few minor differences from the above sequence. The above sequence data for *K. pneumoniae* are identical to those in a report of the sequence of the same region¹⁸, in which the location of the promoter was not determined.

with their regulatory proteins have been conserved as a package in evolution. The *K. pneumoniae nifA* protein is evolutionarily related to the *glnG* product⁴, and preliminary data from our laboratory suggest that the *R. meliloti* 'N' protein is closely related to the *GlnG* protein (V.S., D.W.O. and F.M.A., in preparation; W. Szeto and L. Zimmermann, in preparation). The synthesis of this *R. meliloti* regulatory protein must be

under some form of symbiotic control, to account for the inability of *R. meliloti* to derepress its nitrogenase genes *ex planta*.

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The seal myoglobin gene: an unusually long globin gene

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The tetrameric haemoglobins of vertebrates are encoded by α - and β -globin gene families which arose during evolution by a succession of gene duplications, commencing with an $\alpha\beta$ globin gene duplication which occurred about 500 Myr ago, early in the evolution of the vertebrates^{1–3}. All functional α - and β -globin genes analysed so far share common features, including coding sequence homologies and the presence of two introns within the coding sequence at locations which correlate with interdomain boundaries within globin polypeptides^{4–7}. Here we describe the isolation and characterization of an additional diverged member of the globin gene family, the seal myoglobin gene. We show that monomeric myoglobin, which diverged from haemoglobin about 600–800 Myr ago before the appearance of tetrameric haemoglobins^{8,9}, is also specified by a gene containing two introns at positions precisely homologous to haemoglobin introns. Unlike vertebrate haemoglobin genes, however, the noncoding regions of the seal myoglobin gene are remarkably long.

Diving mammals such as whales and seals have higher levels of skeletal muscle myoglobin than terrestrial mammals^{10,11} and are potentially rich sources of myoglobin mRNA. Previously, we have cloned a partial cDNA copy of myoglobin mRNA from the grey seal (*Halichoerus grypus*)¹². This clone, termed pSM 178, contained the terminal 293 base pairs (bp) of 3'-untranslated sequence in myoglobin mRNA and was used to show that seal myoglobin is specified by a single gene which is transcribed to give an unusually long mRNA, about twice the length of α - and β -globin mRNAs.

To isolate the grey seal myoglobin gene, *Sau3A* partial digests of grey seal DNA were cloned into the *Bam*HI replacement phage vector λ L47.1 (ref. 13) and recombinants screened by hybridization with myoglobin cDNA from pSM 178. Two strongly hybridizing recombinants, termed λ SM.1 and λ SM.19, were isolated and shown by restriction endonuclease mapping to be overlapping isolates of a single region of the seal genome (Fig. 1). The 3' end of the myoglobin gene was located by Southern transfer hybridizations of λ SM.1 and λ SM.19 with cloned myoglobin cDNA. The restriction map around this cloned region corresponds exactly to the map of the seal myoglobin gene previously determined by Southern transfer analysis of seal genomic DNA probed with myoglobin cDNA¹².

Additional potential myoglobin coding sequences were located by hybridizing λ SM.1 and λ SM.19 DNA with ³²P-labelled seal muscle poly(A)⁺ RNA containing high levels of myoglobin mRNA¹². Three hybridizing regions, including the 3' end of the myoglobin gene, were located and subcloned into the plasmid pAT 153 (ref. 14) (Fig. 1). The first two hybridizing

regions were isolated (as a 970-bp *Hind*III DNA fragment and a 1,630-bp *Bam*HI-*Eco*RI DNA fragment, respectively; see Fig. 1), labelled with ³²P by nick-translation and hybridized to seal genomic DNA digested with various combinations of *Bam*HI, *Bgl*II, *Eco*RI and *Hind*III. One major hybridizing fragment was detected in each digest, and these myoglobin DNA fragments could be arranged into a genomic map indistinguishable from the map of the cloned myoglobin gene (data not shown). We conclude that the seal myoglobin gene has been isolated without any major rearrangements.

These three hybridizing regions were completely sequenced (Figs 1, 2). Each region contains myoglobin coding sequences and together they constitute a complete myoglobin gene 9,200 bp long. The amino acid sequence deduced from the exon coding regions corresponds exactly to the sequence reported for myoglobin from the harbour seal, *Phoca vitulina*¹⁵. The coding sequence is interrupted by two very long introns of 4,800 and 3,400 bp at codons 31 and 105–106, respectively. Published alignments of myoglobin with α - and β -globin amino acid sequences¹ show that these intron positions in myoglobin are precisely homologous to those in α - and β -globin genes².

The start of myoglobin mRNA was located within the DNA sequence by S₁ nuclease protection mapping¹⁶ (Fig. 2). A cluster of three potential capping sites was identified; this is preceded, at a distance of 26–28 bp, by a conventional TATA box, as expected for a gene transcribed by RNA polymerase II¹⁷. The CCAAT promoter element frequently found ~80 bp before the capping site of haemoglobin and other genes² is not evident near the seal myoglobin gene; this region is instead occupied by an unusually purine-rich sequence (Fig. 2).

The third exon of the myoglobin gene is remarkably long and comprises codons 106–153 plus 548 bp of 3'-untranslated sequence. This 3' region is present as a continuous sequence in mature myoglobin mRNA, as shown by electron microscopic R-loop analysis of seal muscle poly(A)⁺ RNA hybridized to λ SM.1 DNA, and by S₁ nuclease protection mapping of this region with seal poly(A)⁺ RNA (Fig. 2; data not shown).

The three exon, two intron organization of the seal myoglobin gene is identical to that of vertebrate α - and β -globin genes. This structure is therefore ancient and must have been established before the myoglobin-haemoglobin divergence 600–800 Myr ago. The only exception to this structure is found in the plant globin genes specifying soybean leghaemoglobins, which code for monomeric myoglobin-like proteins yet contain an additional intron within the central globin exon which encodes the haem-binding site^{18–21}. It is unknown whether this extra plant intron was gained recently in evolution by insertion into a globin-like gene, or whether a primordial gene ancestral to globin and leghaemoglobin contained three introns, one of which was eliminated early in animal evolution.

The ancient structure of animal globin genes, together with correlations between globin exons and protein domains^{4–7}, supports the idea that globin genes arose early in evolution by the shuffling and reassortment of unrelated exons^{22–25}. In this context, it is worth noting that some exon-domain correlations, such as $\alpha_1\beta_1$ contact residues specified by exon 3 and Bohr effect residues coded by exons 1 and 3 (ref. 5), relate only to tetrameric haemoglobins and must have evolved long after the basic exon-intron structure of globin genes had been established.

Gene lengths as well as intron positions show a surprising evolutionary stability in haemoglobin and leghaemoglobin genes (Table 1); 5'- and 3'-untranslated regions in α -globin, β -globin and leghaemoglobin genes show little variation in length, as does the first intron in these diverged genes. This apparent conservation has led to the suggestion that intron length, rather than sequence, is implicated in some way in globin gene expression²⁶. It is therefore remarkable that the introns and 3'-untranslated region of the seal myoglobin gene are all very long and combine to make this gene the longest globin gene yet described. Clearly, short noncoding regions are not an absolute prerequisite for globin gene function. It remains

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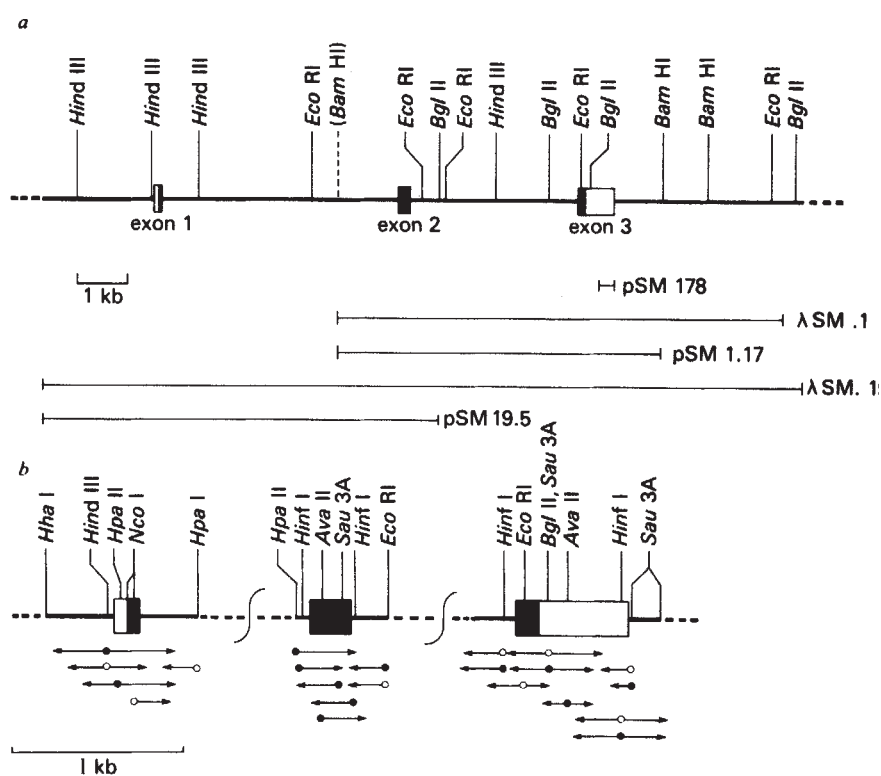
Table 1 Lengths (in bp) of noncoding regions in α -globin, β -globin, leghaemoglobin and myoglobin genes

Gene		5'-NT	Intron			3'-NT	Ref.
			1	Central	2		
α-Globin-related							
Man	$\alpha 2$	37	117	—	140	109	27
Mouse	α	34	122	—	135	103	28
Goat	α^I	39	108	—	103	95	29
<i>Xenopus laevis</i>	α^1	40	171	—	338	109	*
β-Globin-related							
Man	β	50	130	—	850	132	30
	δ	53	128	—	889	129	31
	γ^A	53	122	—	866, 876	88	32
	γ^G	53	122	—	886, 904	88	32
	ϵ	53	122	—	850	122	33
Mouse	β_d^{maj}	52	116	—	653	130	34
	β_d^{min}	52	116	—	628	130	34
Rabbit	β^1	53	126	—	573	92	35
Goat	β^A	52	128	—	906	127	36, 37
	β^C	52	132	—	847	127	36, 37
	γ	52	129	—	827	128	36, 37
<i>X. laevis</i>	β^1	45	192	—	841	130	*
Leghaemoglobin							
Soybean	Lba		119	233	680	174	21
	Lbc ₁		169	234	285	144	21
	Lbc ₂		119	190	197		20
	Lbc ₃	49	119	99	229		19
Myoglobin							
Grey seal	Mb	70-72	~4,800	—	~3,400	548	

NT, untranslated mRNA sequence. The central intron is present only in leghaemoglobin genes and the two sizes of intron 2 in human γ -globin genes represent allelic length variants. Preliminary comparisons of the seal myoglobin gene with human α - and β -globin genes show no significant sequence homologies between the noncoding regions of these genes, as expected for genes which diverged 600–800 Myr ago^{2,8,9}.

* R. M. Kay, R. K. Patient and J. G. Williams, unpublished data.

Fig. 1 Isolation and characterization of the grey seal myoglobin gene. Grey seal DNA¹² was partially cleaved with *Sau*3A and 10–20-kilobase (kb) DNA fragments were recovered, ligated into the *Bam*HI replacement vector λ L47.1, packaged *in vitro* and used to infect *Escherichia coli* WL87 (*rec*⁺)³⁸. 460,000 recombinant plaques were screened by hybridization³⁸ with ³²P-labelled grey seal myoglobin cDNA¹² (isolated as a 642-bp fragment from a *Hha*I digest of the recombinant plasmid pSM 178). Two strongly hybridizing plaques (λ SM.1 and λ SM.19) were purified by two cycles of re-screening on *E. coli* ED8910 (*recBC*) and phage DNA prepared³⁸. *a*, A *Bam*HI, *Bgl*II, *Eco*RI and *Hind*III restriction map of the seal myoglobin gene was determined from λ SM.1 and λ SM.19 DNA. The bracketed *Bam*HI site was created at the left-hand end of λ SM.1 during cloning and is absent from λ SM.19 and seal genomic DNA. The 3' end of the myoglobin gene was located at the indicated position by Southern blot hybridization with ³²P-labelled pSM 178 DNA. Additional coding regions were identified by hybridization with seal muscle poly(A)⁺ RNA labelled *in vitro* with ³²P using T4 polynucleotide kinase and [γ -³²P] ATP³⁹. Three hybridizing regions including the 3' end of the myoglobin gene were detected and more precisely located to the boxed segments by detailed analysis of subclones of λ SM.1 and λ SM.19 in pAT 153 (ref. 14) (plasmids pSM 1.17 and pSM 19.5, respectively). *b*, The three hybridizing regions, corresponding to the three exons of the myoglobin gene, were mapped in detail in pSM 1.17 and pSM 19.5 DNA and subjected to sequencing by the Maxam–Gilbert technique⁴⁰. The sequencing strategy shown used DNA fragments which had been kinase-labelled at the 5' terminus (open circles) or fill-in labelled at the 3' terminus (closed circles). The sequences obtained (Fig. 2) identified myoglobin coding sequences (solid boxes) and untranslated myoglobin mRNA sequences (open boxes). Exon 1 was sequenced from pSM 19.5 and exons 2 and 3 from pSM 1.17.



Northern blot

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Is there evidence for universal rotation?

BIRCH¹ has recently claimed that radio sources in the two hemispheres of the sky are twisted in opposite senses and cites this as evidence for a universal vorticity. His evidence comes from direct measurements of maps (primary measure) of a sample of 30 sources and from measurements of the direction in which the average polarization vector is rotated from the source axis (secondary measure) in a larger sample of 132 sources. From a statistical analysis of his data we find no evidence for an asymmetry in the sense of twist in the sample for which the primary measure is available, and no evidence that the secondary measure is correlated with the primary measure. We believe that the asymmetry in the sample with secondary measure can be accounted for by a small systematic effect in the correction for Faraday rotation in the local interstellar medium.

The first sample is the more interesting one, as a dipolar distribution of radio source twists could be construed as direct evidence for a universal vorticity of intergalactic media. As we discuss below, source-averaged polarization does not seem to have any simple physical interpretation, and as the measurements are subject to large corrections, selection bias and slight systematic errors are a worrying possibility. Consider the sample of 30 with a primary measure (Table 2 in ref. 1). With Birch's choice of pole, the distribution of signum (Δ_i) is: 3+ and 1- in one hemisphere and 14- and 12+ in the other. Measure the degree of asymmetry by an asymmetry parameter

$$a = [N(+)_1 - N(-)_1] + [N(-)_2 - N(+)_2] \quad (1)$$

where $N(+)_1$ is the number of Δ_i s with positive sign in the first hemisphere, and so on. We have performed simulations in which to each source in the sample we randomly assigned a sign of Δ_i , then searched for the pole position which maximized the asymmetry parameter a . The largest possible asymmetry parameter in the Laing sample is 14, obtained when the pole is at $\alpha = 15^\circ$, $\delta = 0^\circ$. The cumulative frequency distribution in a resulting from 1,000 simulations is shown below:

A	4	6	8	10	12	14	16	18	20
$P(a \geq A)$	1.00	0.99	0.92	0.70	0.42	0.19	0.07	0.03	0.01

for 30 sources on a sphere at the positions of those in the Laing sample.

Since a degree of asymmetry as large as that of the observed sample results by chance 19% of the time we are unable to reject the null hypothesis that the sources have random senses of twist. Birch's choice of pole gives $a = 4$. Because Birch

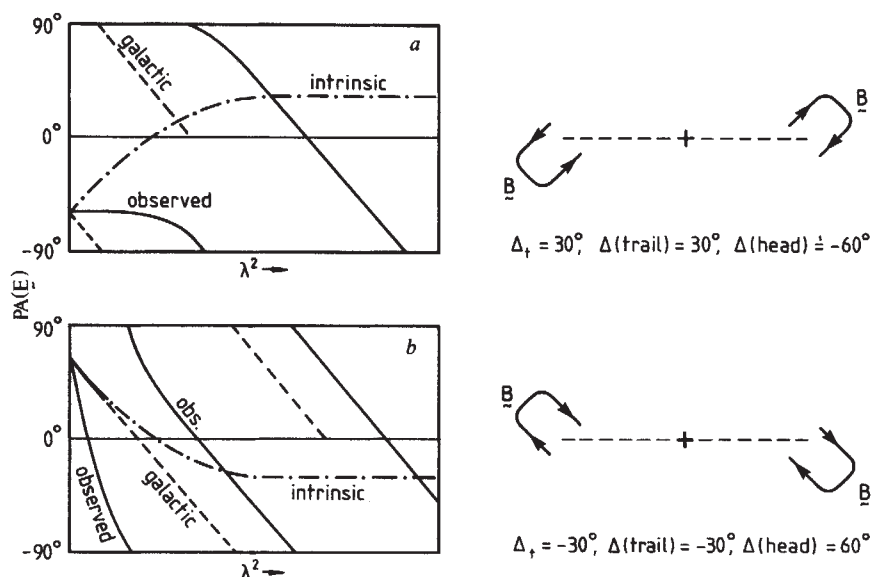


Fig. 1 Mirror-S (a) and S (b)-shaped sources, seen through a medium causing negative rotation. The magnetic fields are as shown. The head is assumed to dominate the flux at short wavelengths and the trail to dominate at long. A single-source model seeking the smallest consistent RM weights the head (negative Δ) in a and the trail (also negative Δ) in b resulting in a correlation between the sign of Δ and RM, independent of source shape.

has already fixed the pole by other considerations, the probability that this occurs by chance is 59%.

Most of Birch's data are for a sample for which only Δ (the angle between the averaged polarization $\mathbf{B}$ vector and the source axis) is available. To admit these data into a discussion of the sense of twisting of radio sources, one must establish that it does in fact correlate with the primary measure. Evidence for this can be sought in the Laing sample of 30 sources for which both measures are available. In 17 cases, the two measures agree on the sign of Δ , and in 13 they disagree. Choosing the relative signs on the basis of coin tosses would give 17 or more agreements 29% of the time. This is not encouraging. A more sophisticated analysis, retaining the magnitude of Δ and Δ_i , must take into account the circular nature of the data. Application of the circular-circular rank correlation procedure due to Mardia^{2,3} yields a positive correlation—at the 55% level (the negative correlation is even less significant). Therefore, we believe that the evidence for a correlation between the two measures is insufficient to justify use of the polarization data as a diagnostic of radio source shape.

There remains the question of a possible asymmetry in the polarization data itself, regardless of its relevance to a discussion of radio source shapes. As the Birch sample of 45 sources is confined to a narrow declination range, we may treat the data as a sequence of pluses and minuses around a circle (as did Birch in his analysis). The correct null hypothesis is that the signs are chosen at random, with equal probability. We have per-

formed simulations in which we gave 46 points spaced uniformly round a circle random signs and found the division (into halves) of the circle which gave the maximum asymmetry parameter a (defined in (1)). The cumulative frequency distribution in a based on 10,000 simulations is shown below:

A	2	4	6	8	10	12	14
$P(a \geq A)$	1.000	0.999	0.974	0.878	0.705	0.500	0.312
	16	18	20	22	24	26	28
$P(a \geq A)$	0.179	0.094	0.040	0.017	0.006	0.002	0.001

for 46 sources equally spaced around a circle.

The asymmetry parameter of the Birch sample taken at face value is 25. Values as large as this arise by chance only 0.2% of the time. A more representative estimate of the probability of the observed sequence should take into account errors in the determination of the sign of Δ . Birch¹ estimates the error in individual Δ s to be 10° (a value we have confirmed by comparing his values with independent determinations of Δ)⁴⁻⁶. In his sample, there are eight values of Δ within 10° of 0° or -90° , and whose sign is therefore uncertain. Consider the 2^8 possible sequences in which the signs of these Δ s are allowed to vary. If each of these is treated as an equally likely representative of the data, the average probability of obtaining an asymmetry parameter larger than that of each sequence is 3%. An alternative test for correlation which retains the values of Δ has been formulated by Mardia³. The source RAs are ranked circularly and the corresponding Δ values linearly. Omitting the eight uncertain Δ s, RA and Δ are found to be

correlated at the 5% level, using Mardia's statistic. The asymmetry may still, therefore, be considered significant. The first exercise, however, provides a warning of the tremendous sensitivity of the probability of obtaining a sequence to tiny systematic errors in Δ and to the selection of sample points, if the selection procedure prefers one type.

There is evidence within the data for such a systematic effect (error or selection bias) in the correction for galactic Faraday rotation. In Birch's sample, there are 18 sources with rotation measure $|RM| > 25 \text{ rad m}^{-2}$ (R. G. Conway, personal communication) (more than 45° rotation at 18-cm wavelength). Of the 15 sources for which the total polarization angle is consistent with that of the two lobes, Birch's Δ has the same sign as rotation measure for 13. This much coincidence would arise by chance 0.4% of the time. Furthermore, the hemispheric split between positive and negative Δ s in the total sample is also that which separates large positive from large negative rotation measures.

The physical significance of Birch's 'average magnetic field' (weighted by polarized intensity) is not completely clear. Most of the 3C sources are extended doubles with bright hotspots⁷. The magnetic fields in hotspots are generally 'wrapped around the head'⁸⁻¹⁰, that is, perpendicular to the direction of elongation at the head [which is often depolarized (R. G. Conway, personal communication) and dominates at high frequency^{8,10}] and parallel to the direction of elongation in the tail. Depending on the relative brightnesses of head and tail in a given source, Birch's average may pick out one or the other feature. This probably explains the lack of correlation between Δ and Δ_t . For example, polarization-resolved maps⁸ indicate that the head dominates in 0210+86 ($\Delta_t = 30^\circ$, $\Delta = -52^\circ$) while the tail dominates in 1845+79 ($\Delta_t = -26^\circ$, $\Delta = -26^\circ$).

Given these properties of radio sources, one can easily imagine ways in which the small correlation with rotation measure could have been produced: for example see Fig. 1. We consider two sources with components separated along PA = 90° , because most of the sources are aligned this way (evidence not of universal shear, but of the east-west orientation of the Cambridge interferometer, which results in higher resolution in the east-west direction than in the north-south direction). When two sources with opposite Δ_t are viewed through a medium giving large negative rotation, a conservative rotation-measure fitting program which seeks the smallest consistent rotation measure will weight the head ($\Delta < 0$) of the source with $\Delta_t > 0$, and weight the tail ($\Delta < 0$) of the one with $\Delta_t < 0$, giving a correlation of Δ with RM. It is difficult to determine how much this effect may have influenced the data, but if the signs of Δ of 30% of the

sources with $|RM| > 20$ are reversed, the asymmetry parameter of Birch's sample drops to 16, which is 18% probable.

Alternatively, if sources with Δ (trail) of opposite sign to RM were eliminated from the sample because of the large curvature in their RM against λ^2 plots (Fig. 1a), and those with the same sign (and smaller curvature, Fig. 1b) retained, a correlation would also be introduced into the data. Birch rejected 10 sources with 'ambiguous' rotation measure fits. If they were omitted for this reason, the true asymmetry would have been 15 in 55, which arises by chance 38% of the time.

We conclude that the data presented by Birch are insufficient to substantiate his claim.

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BIRCH REPLIES—The statistical evidence for universal vorticity¹ depends primarily on the magnitude and sign of Δ , and not, as Phinney and Webster assert, on the handedness of the elongation of the tails of radio sources. The quantity Δ , which is the angle between the average magnetic field and the major axis of a radio source, was found to be predominantly positive in one half of the sky and negative in the other; as agreed by Phinney and Webster, this result had a chance probability for the PB sample of 0.25%. Using the magnitudes of Δ , and vector methods which allow fully for uncertainties in values near 90° , the chance probability becomes 1.2×10^{-6} .

This remarkable result prompted the analysis of the independent samples of Laing, Ekers and Conway, for which the predicted values of Δ were obtained; the significance of this confirmation for the independent Laing sample was 5.2×10^{-3} . Subsequently the model was tested successfully against the directions of the tails of radio sources. There can be no question of the significance of these results, although the secondary result for Δ_t is clearly less firm than the primary result of anisotropy in Δ itself.

The suggested explanation of this in terms of Faraday rotation in the interstellar medium deserves attention. This requires a detailed analysis of the rotation measures for these radio sources. For the Jodrell Bank sample the rotation measures will be published by Conway *et al.* These data show no correlation between the signs of Δ and RM to the 33% level, but there would have to be a high degree of correlation if such an explanation were correct. Furthermore, large errors of $+37 \text{ rad m}^{-2}$ and -19 rad m^{-2} would be required to produce the effect—far larger than the present uncertainties.

It may be of interest that, taking values from ref. 2 for the sample of 94 from which the PB sample of 45 was drawn and not using Jodrell data, the sample can be split into equal halves at RA 0800/1626 to yield:

$$\sin 2\Delta_1 \approx -0.393 \pm 0.088$$

$$\sin 2\Delta_2 \approx +0.097 \pm 0.115$$

These belong to different populations at the significance level $\alpha \approx 2.9 \times 10^{-3}$, and are non-zero with $\alpha \approx 3.3 \times 10^{-5}$.

The evidence I adduced remains strong and unexplained except by universal rotation; that is, the statistical significance is high and the suggested systematic errors cannot adequately explain the data.

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Corrections to the nucleotide sequence of the src gene of Rous sarcoma virus

"There's always wan encouragin' thin' about th' sad scientific facts that come out ivry week in th' papers. They're usually not true." (ref. 1)

WE previously reported a nucleotide sequence for the v-src gene and its environs in the genome of the Schmidt-Ruppin strain of Rous sarcoma virus (RSV)². Subsequent unpublished work by ourselves and other investigators revealed the likelihood of errors in our original report. We therefore reinvestigated the suspect regions of the sequence and indeed discovered mistakes in our previous data. The errors were detected by (1) restudy of original autoradiograms, (2) reference to detailed restriction maps of molecularly cloned DNA and (3) resequencing of the regions of greatest doubt. Here we report the important changes necessitated by our new data (see Fig. 1). A complete version of the revised

PstI - 7%
 CTCGACGACGCTGAGCTGACTGCTGCTGCTGCGCTGCGCTGACACTGTTGCGACGCGCGTAGCTGGGACGTGCA
 +127
 Arg Ser Leu Glu Pro Asp Ser Thr His His Gln Gly Phe Pro Ala Ser Gln
 GCG ACC CTG GAG GCA GCG GAC AGC AAG Ser AGC AAG OCT AAG GAC CCG AGC CAG CCG CCG
 Thr Pro Asn Lys Thr Ala Ala Pro Asp Thr His Arg Thr Pro Ser Arg Ser Phe
 ACC CCG AAC AAG ACA ACA GCG CCG GAC AGC CAC CCG CCG ACC CCG AGC CCG TCG TTC
 Gly Thr Val Ala Thr Glu Pro Lys Leu Phe Gly Lys Phe Asn Thr Ser Asp Thr
 CCG ACC GTG GCG ACC GAG CCG AAG CTC TTC GCG GCG PTC AAC ACT TCT GAG ACC
 Val Thr Ser Ser Pro Gln Arg Ala Gly Ala Leu Ala Gly Gly Val Thr Thr Phe Val
 GTT ACG TCG CCG CAG CCG GCG GCG GCA CTC GCT GCG GCG GTC ACC ACT TCT GTG
 Ala Leu Thr Asp Thr Gly Ser Trp Ile Glu Thr Asp Leu Ser Phe Lys Lys Gln
 GCT CTC TAC GAC TAC GAG TCG TCG ATT GAA ACG GAG TTG TCG TTC AAG AAA TGA
 Gly Arg Leu Gln Ile Val Asn Asn Thr Gly Lys Thr Trp Leu Ala His Ser
 GAA CCG CTC CAG ATT GTC AAC AAC ACG GAA GGT AAC TCG TCG CCG OCT ACT TCG
 Leu Thr Thr Gly Gln Thr Gly Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ser Asp
 CTC ACT ACA GGA CAG ACG GCG TAC ATC CCG AGT AAC TAT GTC CCG CCG TCA GAC
 Ser Ile Gln Ala Glu Glu Trp Thr Phe Gly Lys Ile Thr Arg Arg Gly Ser Gln
 TCC ATC CAG OCT GAA GAG TGG TAC TTT GCG AAG ACT ACT CCG CCG GAG TCG GAG
 Arg Leu Leu Leu Asn Pro Glu Asn Pro Arg Gly Thr Phe Leu Val Arg Glu Ser
 CCG CTC CTC CTC ACG CCG GAA AAC CCG CCG GGA ACC TTC TTG GTC CCG GAG ACG
 Glu Thr Thr Ala Lys Gly Ala Thr Cys Leu Ser Val Ser Asp Phe Asp Asn Ala Lys
 GAG ACG ACA AAA GGT GCG TAT TCG CTC TCG GTT TCT GAG TTT GAG AAC GCG AAG
 Gly Leu Asn Val Lys His Thr Lys Ala Ile Arg Lys Leu Asp Ser Gly Phe Thr
 GCG CTC AAT GTG AAG CAC TAC AAG ATT CCG AAG CTC GAG AGC GCG GCG TTC TAC
 Ile Thr Ser Arg Thr Gln Phe Ser Ser Leu Gln Gln Leu Val Ala Tyr Thr Ser
 ATC ACC TCA CCG ACA CAG TTC ACG ACG CCG CTC CAG CAG CTC GTG CCG TAC TAC TCG
 Lys His Ala Asp Asp Gly TCG Cys His Arg CCG CTC AAC Val Cys Pro Thr Ser Lys
 AAA CAT GCT GAT GCG TTG TGC CAC CCG CTC ACC AAG GTC CCG CCG CCG TCG AAG
 Pro Gln Thr Gln Gly Leu Ala Lys Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu
 CCG CAG ACC CAG GGA CTC GCG AAG GAC CCG TCG GGA AAT ATC CCG CCG GAG TCG CTC
 Arg Leu Glu Val Lys Leu Gly Gln Gly Cys Thr Gly Gly Val Trp Met Gln Thr
 CCG CTC GAG GTG AAG CTG GCG CAG GCG TCG TTT GGA GAG GTC TCG ATT GCG ACC
 Trp Asn Gly Thr Thr Arg Val Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Ser
 TCG AAC GCG ACC ACG ACA GTG CCG ATA AAG ACT CTG AAG CCG CCG ACC ATG TCG
 Pro Ala Ala Phe Leu Gln Glu Ala Gln Val Met Lys Lys Leu Arg His Gln Lys
 CCG GAG CCG TTC CTC CAG GAA CCG CAA GTG ATG AAG AAG CTC CCG CAT GAG AAG
 Leu Val Gln Leu Tyr Ala Val Val Ser Glu Glu Pro Ile Tyr Ile Val Ile Gln
 CTC GTT CAA CTG TAC GCA GTG GTG TCG GAA GAG CCG OCT ATC TAC ATC GTC ATT GAG
 Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys Gly Glu Met Gly Lys Tyr
 TAC ATG AGC AAG CCG AGC CTC CTC GAT TTC CTC AAG GGA GAG ATG CCG AAG TAC
 Leu Arg Leu Pro Gln Leu Val Asp Met Ala Ala Gln Gln Ile Ala Ser Gln Met Ala
 CTC CCG CTC CCA CAG CTC GTT ACT ATG GCT GCT CAG ATT GCA TCG GCG ATC GCG
 Tyr Val Glu Arg Met Asn Tyr Val His Arg Asp Leu Arg Ala Ala Asn Ile Leu
 TAT GTG GAG ACG AAT AAC TAC CTC CAG CCA GAG CCG CTC CCG GCG ACC AAT ATC Leu
 Val Gly Asn Leu Lys Cys Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile
 GTG CCG GAG AAC CTC GTG TCG AAG GTG GCT GAC TTT GCG CTC GCA CCG CTC ATC
 Glu Asp Asn Gly Tyr Thr Ala Arg Gln Gln Ala Lys Phe Pro Ile Lys Trp Thr
 GAG GAC AAC GAG TAC ACA CCA CCG CAA GTT GCG AAG TTC CCG ATC AAG TCG ACA
 Ala Pro Glu Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser
 CCG CCG GAG GGA CCG CTC TAT CCG CCG TTC ACC ATC AAG TCG GAT GTC TCG TCG
 Phe Gly Ile Leu Thr Thr Glu Leu Thr Thr Lys Gln Arg Val Pro Tyr Pro Gly
 TTC GCG ATC CTC CTC ACT GAG CTC ACC ACC AAG GCG CCG GTG CCA TAC CCA GCG
 Met Gly Asn Gly Glu Val Leu Asp Arg Val Glu Arg Gly Tyr Arg Met Pro Cys
 ATG CCG AAC CCG GAG GTG CTC GAG CCG GTG GAG AGC GCG TAC CCG ATC CCG CCG
 Pro Pro Glu Cys Pro Gln Ser Leu His Asp Leu Ser Cys Gln Cys Trp Arg Arg
 CCG CCG GAG TCG CCG GAG TCG CTC CAT GAC CTT ATG TCG CAG TCG TCG CCG ACG
 Asp Pro Glu Glu Arg Pro Thr Phe Gly Tyr Leu Gln Ala Gln Leu Leu Pro Ala
 GAC OCT GAG GAG CCG CCG ACT TTC GAG TAC CTC CAG CCG CAG CTC CTC OCT GCT
 Cys Val Leu Gln Val Ala Gln (524) Glu (522) +1522
 TGT GTG TTG GAG GTC GCT GAG TAG TCGCGAGTAAATTTTAAAGCTACACAAGGCAAGCGCTT
 +1480 d.f.
 GACGACAAATTCATGAAGAACTCTCTTACGTTTACGCTTTTTCGCTCTCTTTCGAGTGTACCGGCGCAGAT
 d.f.
 ATACCGGTATCTGAGCGGACGACTAGGCTGTGTTTATAGCGGAAAGCGCGGCTTCGGTTGTACCGCGTTACGAGT
 d.f.
 CCGCTTAGGATATAGTAGTTTTCGCTTTTTCGATAGCGGCGAAATTTAGTCTTATCGAATACCTTTTATAG
 TCTTGCAACATCGTAACTGATGAGTTAGCAACATCGCTTACAAAGGAGAGAAAAAGCAGCGTGCATTCGCGATT
 PvuI
 GGTGGAATTAAGGTGTGATGATGCTGCTTATTAGGAAGGCAACAGAGCGGCTCTGACATGGATTGTGAAGAA
 EcoRI
 CCACTGAATTC- +1785

The length of the protein has been changed from 530 to 526 residues. For-

tuitously, the revisions have no other appreciable effect on previously recognized important features of the amino acid sequence. Codon usage remains essentially as described before². Serine remains at residue 17, a position that we have identified as the major site for phosphorylation of pp60^{v-src} by cyclic AMP-dependent protein kinase (unpublished findings of J. Smart, H. Oppermann and J.M.B.). As noted previously³, the major site of tyrosine phosphorylation in pp60^{v-src} has been relocated from residue 419 to residue 416, but the adjoining amino acid sequence is unaltered for more than 40 residues on either side of the tyrosine. Sites for preferred cleavage by the V8 protease of *Staphylococcus aureus* and other proteases can still be identified in predicted regions of the sequence². The chemical composition of the protein remains generally hydrophobic, and the amino acid sequence contains no recognizable domain by which the protein might insert into the plasma membrane of the host cell⁴. The revised sequence still displays provocative homologies with the amino acid sequences of other protein kinases, including the lysine at residue 295 that has been suggested by analogy to be the site of binding for nucleoside triphosphates⁵.

Our previous report proposed an amino acid sequence for the protein gp37, encoded by the *env* gene of RSV. We have located an error that introduced a frameshift near the carboxy-terminus of the amino acid sequence. The revised sequence is shown in Fig. 1. The revision changes the last 6 residues of the previous version of the sequence and extends the protein by an additional 15 amino acid residues. The carboxy-terminal domain remains hydrophobic², however, as required by previous proposals that this portion of the molecule might be inserted into the lipoprotein envelope of the virus⁶.

We have also found that our original version of the nucleotide sequence between *env* and *v-src* required revision by both the removal and addition of nucleotides. Figure 1 illustrates the two domains of the sequence where substantial changes were necessary. The revisions relocate by a few residues, but do not change the composition of either the splice acceptor site used in the genesis of *src* mRNA (marked in Fig. 1) or the large direct repeat that brackets *v-src* (as described in ref. 2). The entire untranslated sequence extending from the end of *v-src* to the 3' end of the viral genome remains as reported by us previously^{2,7}, with the exception of one nucleotide which is indicated in Fig. 1.

Our revised sequence is now in general accord with all data available to us from other workers on several strains of avian leukosis and sarcoma viruses (although differences between single nucleotides still exist, as might be expected for different strains). We can offer no single

explanation for the errors in our previous data; we thank several colleagues who brought to our attention the discrepancies between their results and ours and deeply regret that our errors found their way to print; we apologize to any investigator who may have been inconvenienced by the mistakes in our previous publication.

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Mean IQ differences in Japan and the United States

IN Lynn's article¹ on Japanese-US mean IQ differences, it is stated that "whereas Americans . . . have ~2% of their populations with IQs over 130, the Japanese have ~10% at this level. Among the population as a whole, 77% of Japanese have a higher IQ than the average American . . ." These calculations assume, however, that the respective variances of the American and Japanese IQ distributions are identically equal to 15 (and, of course, that the distributions are normal). My reading of the statistical source from which most of Lynn's data are obtained, namely, the Japanese and US WISC-R manuals^{2,3}, suggests that, in fact, the Japanese IQ variance is significantly lower. The Japanese manual (Table 3, p. 26) gives a standard deviation of the sum of scaled scores on the performance test which is 85.4% of that of the American sum of scaled scores on the same test (see Table 6, p. 23, in the American manual).

The standard deviations reported in the two manuals are comparable (that is, are in the same units) if the linear functions used to translate the raw scores into scaled

scores in the two standardizations have the same slopes, as Lynn himself implicitly assumes in his calculations. Let $Y_U = a_U + bX_U$ and $Y_J = a_J + bX_J$, where Y_U and Y_J are the scaled scores on a given test for the US and Japanese samples, respectively, and X_U and X_J are the raw scores for the two samples. Only the latter are comparable, because only they are measured in the same units. Because $E(Y_U) = E(Y_J)$, by construction, $E(X_J) - E(X_U) = \frac{1}{b}(a_J - a_U)$. It is the latter which Lynn estimates, reported in the standard deviation units of X_U . In addition, the ratio of the standard deviations of X_U and X_J will be the same as the ratio of the standard deviations of Y_U and Y_J only as long as b in the two transformation equations is the same, since $\sigma_{Y_U} = b\sigma_{X_U}$ and $\sigma_{Y_J} = b\sigma_{X_J}$.

Assuming that the slopes of the transformation equations are the same, our best estimate of the standard deviation in Japanese IQ scores is 85.4% of the American standard deviation of 15, or 12.8. The proportion of Japanese scoring above 130 (on the American scale) would be, for this estimate, 6.9% rather than 10%, given a Japanese mean IQ of 111 (relative to the American mean of 100). The proportion of Japanese scoring above the American mean would be 80% rather than 77%. A calculation of the two points of intersection of the two IQ distributions shows that the relative frequency of Americans exceeds that of the Japanese for all IQs above 177 and below 104. The Japanese are relatively more numerous for all IQs between 104 and 177. These calculations, of course, are only illustrative and change quite sensitively with respect to the difference in mean IQ assumed. For example, if we use Lynn's previous estimate⁴ of the Japanese-US mean IQ gap of 6.8 and still assume a 85% lower standard deviation in Japanese IQs, Americans would be relatively more frequent at all IQs above 158 and below 95, and the Japanese relatively more frequent at all IQs between 158 and 95. The proportion of Japanese scoring above 130 would be 3.5%, as opposed to the American 2.3%.

Freedman provides some interesting speculation as to why we might expect, on genetic grounds, a lower variance in various quantitative traits among the Japanese than among Americans and Europeans⁵.

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2000: A space odyssey

Bernard Lovell

IN THE state of euphoria surrounding the lunar landings of 1969 Wernher von Braun believed that a manned landing on the planet Mars would be the next major space effort for the United States. To launch the increased payload from Earth he proposed to strap solid-fuel rocket motors to the first stage of the Saturn 5 vehicle. For the third stage he planned to replace the hydrogen-oxygen fuel of Apollo by a nuclear engine. If von Braun could have had his way, and if an effort equivalent to that which made the Apollo project possible had been available, it is probable that by now the attempt to land men on Mars would be in progress.

The aftermath of Apollo was altogether different. Von Braun had the chagrin of witnessing the dismantling of many of the space-programme facilities and the dispersion of the men who had made the Saturn rocket a reality. Those who had argued that instrumented landings on the Moon would be cheaper, safer and as good scientifically as manned landings now had their turn. Have the brilliant successes of the Viking landings and orbiters proved the point that there is no case for sending men to Mars? James Oberg soon gives the answer and quotes one of the builders of the Viking probes that "the main purpose is to bring back science, not rocks". On financial issues it is argued that in view of developments since Apollo — particularly the Shuttle — the expense of a man-to-Mars programme would be far less in terms of "real dollars" than the Apollo lunar programme.

The contention of Oberg, and apparently of many other people who assembled on the campus in Boulder, Colorado, in 1981 for a "Case for Mars" colloquium, is that realistic plans can now be made to mount a manned mission to Mars by the end of this century. Even so there seem to be formidable difficulties — but perhaps no more so than those which confronted NASA when President Kennedy pledged the nation to the Apollo programme in 1961.

The energy necessary for the rocket propulsion remains a prime consideration. Using the minimum energy orbit between Earth and Mars (the Hohmann ellipse) involves a space journey of some 200 million miles. With this minimum energy launch window, which occurs every 25 to 26 months, the journey time to Mars would be between 220 and 300 days. Unfortunately the similar minimum energy launch window from the planet to Earth does not occur until 460 days after arrival. Thus the

Mission to Mars: Plans and Concepts for the First Manned Landing. By James E. Oberg. Pp.221. ISBN 0-8117-0432-7. (Stackpole: 1982.) \$14.95.

astronauts would be absent from Earth, either in space or on Mars, for at least 900 days. With any conceivable means of rocket propulsion likely to be available this century, the best compromise would be to schedule the launching to make use of the gravitational attraction of a Venus fly-by on the return journey. By this means the stay on Mars need be only a few weeks and the absence from Earth would be reduced to 600 to 700 days.

Nearly two years of weightlessness with a week or so subject to only 0.38g on Mars presents a number of problems for the human body. Encouragement is derived from the experience of the Soviet astronauts who were able to walk immediately on return to Earth after six months in Earth orbit. Exercise is clearly essential to prevent deterioration of the heart and the bones — every day the Soviet astronauts did two to three hours exercise, equivalent to climbing a 200-storey block. The problem of bone-decalcification is unsolved, however. Under conditions of weightlessness one-half per cent is lost per month and unless this loss rate declines after a few months in space, 10 per cent loss of bone calcium, considered to be very serious, would occur by the time the Mars voyage could be completed. Oberg recognizes that there may be no cure but hopes that some countermeasure will soon be found.

The radiation danger from the Sun and cosmic rays is also freely acknowledged. A "bad" year of solar flares could sicken or kill the crew of the spaceship unless they are protected and it will be essential to build some kind of radiation shelter into the Mars spaceship. Such factors and the need to provide facilities to keep the astronauts in training and to prevent boredom (for example a landing simulator) will influence the design of the spaceship.

As a means of propulsion across interplanetary space, a "light sail", in which solar photons billow a giant metal foil sail, seems unlikely to be developed sufficiently by the end of the century. Some means of electric propulsion is favoured — either ion-drive or plasma drive. NASA tested an ion-thruster in space in 1970 and the Soviets in 1966. The power required will be several hundred kilowatts (the Shuttle has less than 20 kilowatts). The idea of

using a nuclear device to produce this power is no longer favoured because lightweight and highly efficient solar cell arrays have now been developed.

For the 1969 manned lunar programme the Saturn rocket placed about 40 to 50 tons in Earth orbit in order to land the 14-ton Lunar Excursion Module on the Moon. Until the detailed designs of the Mars spaceship are made, only estimates can be made of the tonnage that will need to be placed in Earth orbit. Probably about a thousand tons will have to be launched from Earth and the actual lander on Mars is likely to be four or five times heavier than the lunar module. Hence the Mars spaceship will have to be assembled in a parking orbit around the Earth using a considerable number of Shuttle launchings from Earth. Oberg points out that in a few years NASA plan to place a total of several thousand tons of material in Earth orbit using 20 or more Shuttle missions, and that the Soviets are believed to have developed a rocket capable of lifting 200 tons from Earth. Thus the assembly of a thousand-ton spacecraft in Earth orbit using the systems likely to be available by the end of the century is not regarded as a serious issue.

The arrival at Mars seems likely to be a greater problem since the velocity will have to be reduced by about 6,000 mph over a very short period. The use of forward-facing rockets would be the conventional technique, but this means carrying propellant from Earth to Mars, and "aerocapture" using the Martian atmosphere to slow down the spaceship would be more efficient. The trouble in that case is that the astronauts would be subject to forces of 6 to 8g over a period of a minute. After six to eight months of weightlessness some medical authorities believe this would result in bone fracture.

Of all the hurdles that a man-to-Mars project would have to leap, that of cost is the first and the most formidable. The Apollo programme cost the United States in the order of 25–35 billion dollars, and the figure of 100 billion dollars has often been mentioned as an order of magnitude for the Mars adventure. Oberg rejects this firmly. In an illuminating analysis of the costs he arrives at a figure of 20 billion dollars (1981) and compares this with the Apollo programme which would be 63 billion 1981 dollars and the Shuttle development cost of 14 billion. His discussion of why the United States should proceed with the programme is realistic. No claim is made that the mission will be

solely for science — “but science will be well served”. The political and social issues, and the possibility of eventual colonization all receive attention.

Oberg's analysis of Soviet intentions is important. If Western intelligence is correct — that they have a 14-million-pound thrust booster under test — then a Soviet two-year manned fly-by of Mars and return to Earth cannot be many years away. Only two or three of these rocket launchings would be required to assemble a spaceship in Earth orbit for a Mars landing and the long duration trends of recent Soyuz-Salyut enterprises underlines Oberg's conclusion that they really mean to send men to Mars.

Oberg's book is a timely and valuable document and it is to be hoped that the American space planners will take notice of the well-reasoned discussion. It is perhaps unfortunate that a short section near the beginning, “Scenario”, reads like science fiction, but the remainder of the book is a serious scientific and technical analysis of a great adventure with which man could end the twentieth century. □

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School of leeches

Brian Mulloney

Neurobiology of the Leech. Edited by Kenneth J. Muller, John G. Nicholls and Gunther S. Stent. Pp.320. ISBN 0-87969-146-8. (Cold Spring Harbor Laboratory: 1982.) \$36, (US), \$43.20 (elsewhere).

Neurobiology of the Leech invites immediate comparison with two classics: T.H. Huxley's *The Crayfish* and Eric Kandel's *The Cellular Basis of Behaviour*. All three treat a particular animal as an entry to a broad consideration of biological problems at several levels of analysis, including the development of the animal and the physiological, the structural and the evolutionary bases of its behaviour. *Neurobiology of the Leech* is instructive, therefore, of the perils of editorial committees and multi-author books. It lacks the brilliant style and flashes of insight that Huxley achieved a century ago, and also the thoughtful coherence and intellectual depth that make Kandel's book such a fine contemporary introduction to neurobiology.

The ten chapters review four topics, Sawyer and Payton leading off with an introduction to the structure and behaviour of leeches. Readers should be warned that Sawyer's enthusiasm for Anderson's (1973) phylogeny is not shared

by all zoologists, and not used in most textbooks, even though it may be correct. The book then gets down to neurobiological business with chapters by Blackshaw and by Stent and Kristan that describe some of the sensory and motor neurons in leeches, and describe how the nervous system makes the leech swim. These authors review well work up to 1980, but do not provide the broader, comparative analysis that would make the book suitable for beginners. The analysis in the contribution by Stent and Kristan has also been made obsolete by new results from their own laboratories; the last paragraphs of the chapter hint at the scale of the revision that is necessary.

Synaptic structure and transmitter chemistry are reviewed in two chapters; one is a very thorough analysis by Muller that duplicates his 1979 review in *Biological Reviews*, the other a workmanlike review by Wallace. Together they demonstrate that, at the cellular level, leeches are like any other well-studied metazoan; they have vesicles, transmitters, receptors and gap junctions that can be studied here as well as anywhere. The chapters do not make clear that these results apply beyond this class, and should be known to all of us who study neuronal organization; the results do apply and should be familiar.

The final chapters cover the development of the leech's nervous system in embryos and the regeneration of axons and connections in adults. Weisblat's account of development is a crisp review of the lineage studies and analysis of neurogenesis and differentiation that he and others in Stent's group have conducted. The publisher, however, has served Weisblat poorly; the reproduction of his wonderful photographs achieves the quality of a tabloid on an average day, and the reader will not be able to see how exciting they are. Again, the chapter is devoid of any comparative analysis or perspective that would help naive readers.

These authors have taught a successful course, with the same title, each year at Cold Spring Harbor Laboratories. The course recruits as students advanced graduate students and postdocs, and drills them in the literature and methods of the Nicholls and Stent school. The book succeeds best as a review for the same categories of readers, graduate students revising for examinations and postdocs considering neurobiology as a career. It fails progressively as the target audience broadens, because it lacks any consistent attempt to put the neurobiology in either a comparative or a behavioural context. It is, however, an excellent summary of the activities of the Nicholls and Stent school during the past two decades, and, as a bonus, it includes as appendices a useful manual for electrophysiology of leeches and an atlas of neurons in *Hirudo*. □

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Monkish zealots and dark anathemas

Mark Ridley

The Descent of Darwin. By Brian Leith. Pp.174. ISBN 0-00-219548-8. (Collins: 1982.) £7.95.

If I say that Brian Leith is a BBC producer, and that the subtitle of his book is *A Handbook of Doubts about Darwinism*, most British readers, at least, will be able to guess the contents: the Gouldian synthesis of anti-adaptationism, punctuated speciations, macroevolution, and structuralist embryology, philosophers on tautology, Steele's Lamarckism, and cladism; only Sir Fred is missing. Such are the ideas which, Leith believes, may be about to replace traditional neo-Darwinism. All the doubts, as Leith makes clear, are about natural selection, not evolution.

Brian Leith, the dust-jacket tells us, has “an undying admiration for Darwin, if not [it adds] for some of his disciples”. And one can only sympathize. The traditional Darwinians we meet in these pages are enough to kill off anyone's admiration. They are the most terrible of people, dogmatically devoted to their unbending faith, puffed up and over-confident, now assassinating characters, now stopping up the heretic's funds, now scattering their enemies, driving them to suicide, to destitution, and even to Australia. They are at their worst with Mendelism which, Leith tells us, it is simply not possible to suggest is wrong. No wonder he does not admire them! No wonder he hands down what might otherwise be considered condescending advice! Let the Darwinians clarify what they mean by “random”: let them avoid “sloppy thinking”: let them rid themselves of reductionism.

We must accept, if we are to go on, that this is a partisan book. Then we can ask just how well it succeeds in explaining the controversies to its intended reader, someone who does not know about them already. It is less clear on the purely destructive criticisms of natural selection than on the constructive alternatives such as macroevolution, structuralist embryology and Lamarckism. Thus, the discussion of whether Darwinism is falsifiable could be better, although I was amused by the way Sir Karl is nearly always introduced by some such epithet as “great philosopher” or “leading twentieth-century philosopher of science”. The accounts of punctuated equilibrium, and of Steele's work are generally clear, but the former does give the impression that macromutations (“anathema”, we are told, to the neo-Darwinians) rather than geographic speciation are the preferred explanation. A beginner, I suspect, after reading the chapter on cladism, would not know much about what it actually is; he would really have learned no more than that it is some

kind of taxonomy which does not assume that evolution has taken place.

The book has the journalistic merit of being easy to read. The fluency is gained, I think, by concentrating on who disagrees with the orthodox, and by avoiding much in the way of exposition of ideas. We learn who the orthodox neo-Darwinians disapprove of, but we do not learn why

(except for prejudice) they disapprove. If only Leith had not lost so completely his admiration for those monkish zealots, screaming dark anathemas out of their cells, he might have found that they are capable of reasoning too. □

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Excitement in theory and in practice

R.F. Barrow

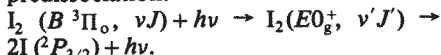
Dynamics of the Excited State. Edited by K. P. Lawley. Pp.667. ISBN 0-471-10059-5. (Wiley: 1982.) £35.90, \$77.50.

ADVANCES in chemical physics, theoretical but more particularly experimental, are leading to the happy conclusion that more and more specialists in different fields talk to each other. The development of laser and molecular beam technologies has resulted in the production of qualitatively new information, not only about the details of stationary molecular energy states but also about the rate constants for the transfer between states under the action of photons or of collisions with other particles. This trend is evident in *Dynamics of the Excited State*, where there is matter to engage the attention of the pure state-to-state kineticist, the specialist in energy transfer, the photochemist, the theoretician and the spectroscopist.

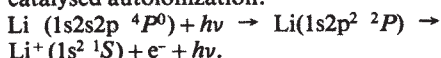
The nine chapters have been written by experts from various fields. Some of the contributions are primarily experimental, dividing into single photon experiments such as the laser-induced fluorescence of the halogens and other diatomic molecules, the quenching of electronically excited metal atoms, the study of photofragment dynamics, and multiphoton processes as represented by a chapter on infra-red excitation and dissociation. Other sections, on the processes of photon-catalysed predissociation and autoionization, on the statistical mechanical treatment of unimolecular reactions induced by infra-red radiation (URIMER!), on rotational to translational and electronic to vibrational energy transfer and on the calculation of potential energy surfaces might be said to be primarily theoretical and interpretative.

The subjects discussed are those that might be expected in a book with this title, and they are treated at an advanced and exciting level. Some contributors have gone beyond the professional review and, for example, much of the material in the chapter on fitting laws for rotationally inelastic collisions is new. All of the accounts are reasonably up to date, but that on the photon-as-catalyst effect describes phenomena that were first proposed and analysed only some five years ago. The iodine molecule (as so often!) provides an example of catalysed

predissociation:



In a second process, the resonant intermediate level might be an unpopulated rotational-vibrational level in the ground electronic state. The lithium atom is used to illustrate the possibility of photon-catalysed autoionization:



This is a well-edited and stimulating book. Overall one gets a good impression of the development of elegant and powerful new experiments; for their interpretation, however, we must await new insights. □

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Imaginative imaging

K.V. Ettinger & U.J. Miola

Imaging with Ionizing Radiations. By K. Kouris, N.M. Spyrou and D.F. Jackson. Pp.204. ISBN 0-903384-30-2. (Surrey University Press/Blackie: 1982.) £29.75.

IT IS A long time since the chest X-ray and celluloid negative of broken bones made their entry into the world of medical diagnostics. With ionizing radiations we map and display hidden anatomical structures, patterns of cell metabolism, the ebb and flow of radioactive tracers, the circulation of blood.

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imaging comes from Kouris, Spyrou and Jackson, all from the University of Surrey. The book is a comprehensive and serious introduction to the physical principles and mathematical tools of imaging, as applied to the needs of nuclear medicine. The mathematical treatment is simple, yet rigorous, with engineering and technological details kept to the minimum.

The introductory chapter links information theory with the process of imaging and image reconstruction. There is some vagueness in the concepts of useful and useless information discussed in that section, arising from the inadequacy of quantitative information theory to deal with the content (i.e. quality) of the message. The discussion of various reconstruction techniques is clear and

adequate, and the main modalities of medical imaging (X-ray radiography and tomography, radionuclide imaging with single photon as well as positron tomography) are included. Use of heavy charged particles for radiography and tomography is also discussed.

Imaging with Ionizing Radiations will serve well as a reference book for the medical physicist and nuclear medicine specialist. Because of its comprehensive exposition of the subject and clear language, it may also find use as a textbook of imaging theory for courses in medical physics. □

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Rules of law in the deep south

Deborah Shapley

Antarctic Law and Politics. By F.M. Auburn. Pp.361. ISBN 0-905838-39-4. (C. Hurst, London/Croom Helm, Canberra/Indiana University Press: 1982.) £19.50, \$32.50. *The New Nationalism and the Use of Common Spaces: Issues in Marine Pollution and the Exploitation of Antarctica.* Edited by Jonathan I. Charney. Pp.343. ISBN 0-86598-012-8. (Allanheld Osmun, Totowa, New Jersey: 1982.) \$39.50.

THE war in the Falklands has drawn world attention to the far south. F.M. Auburn's account of Antarctic law is thus most timely; although his book went to press before the war, Auburn brings up to date (to 1981) the political complexities of the Antarctic and the weaknesses of the region's system of governance under the Antarctic Treaty. The book will doubtless be much cited as 1991, the date the Treaty is to be reviewed, approaches.

Auburn, associate professor of law at the University of Western Australia at Perth, is one of the few lawyers to have made a long-term study of the Antarctic, and his recital of the tangle of national legal conflicts in the region is a good antidote to the commonly held view that Antarctica is a *tabula rasa* on which the nations of the world, or the Antarctic Treaty powers, can write at will. He tours every aspect of Antarctic law — from the authority of the Treaty parties, now numbering 14, to its jurisdiction offshore (a matter of some controversy) — and presents much relevant information about recent events in Antarctica and their legal ramifications. His general conclusion is that the "system" by which the Antarctic is governed is so pitted with legal loopholes that it may be "inadequate" to deal with the new issues of krill and mineral resource exploitation.

There is an especially useful discussion of the legal status of the bases built on the

continent since the Treaty came into force in 1961, and whether they "count" towards sovereignty claims, now or in the future. The Treaty states explicitly that they should not, but Auburn calls the pertinent phrase "an exercise in unreality". Indeed, his lawyer's mind finds problems throughout the Treaty. He asks how the nations involved could renounce "military purposes" in Antarctica (in Article One which says, "Antarctica shall be used for peaceful purposes only") when many of the parties are not renouncing national sovereignty there, and military power is a key ingredient of sovereign power? And how can the Scientific Committee for Antarctic Research (SCAR), the non-government group that coordinates Antarctic research, call itself non-political when a nation's membership of SCAR is a *de facto* prerequisite for achieving consultative status in the Treaty group?

In his suspicions of the Treaty system, Auburn represents a widely held viewpoint. The original parties to the Treaty have strong vested interests, conduct their business in great secrecy and have systematically excluded UN agencies from involvement in Antarctic affairs. All of this has opened them up to understandable criticism for being a closed club.

It is certainly possible, as Auburn does, to pick the Treaty system apart from the standpoint of law and logic; but the law is not the sole cipher by which the realities of Antarctica's governance may be decoded. The "system" is not really based in law although it uses legal means to accomplish many of its goals. Instead, it is based on the peculiar political balance among the odd mix of countries that happen to have permanent commitments to the region. Although each nation became involved for different historical reasons, their interests now lie in the region's resources. The

delicate balance among the group now rests on its wish to draw up a resource regime without a breakdown in the group or a concerted attack by non-parties to the Treaty. The law just doesn't have much to do with all this.

Auburn's treatment almost inspires wonder that the so-called Antarctic system hasn't broken down. By contrast, *The New Nationalism* . . . reflects a more American view: that the Treaty system is really quite stable and proved its worth in 1980 when the new Convention for the Conservation of Antarctic Marine Living Resources — the first major resource regime for the region — was drawn up. This second book, a collection of papers edited by Jonathan Charney, is better than most writing on the Antarctic in that the authors remain generally neutral towards their subject; many people who write about Antarctica have strong views on whether the continent is "common heritage" and on the merits or otherwise of the Treaty.

The collection includes strong papers: on mineral resources and related environmental issues by James H. Zumberge, the President of SCAR; on suggestions for the design of a minerals regime, by Charney; and on the negotiations that led to the living resources convention by James N. Barnes, an adviser to the United States delegation. There are also four papers on global marine pollution. If the Charney collection has a fault, it is a general tendency to take the Treaty system and the region's political stability for granted. Auburn, for instance, makes a good point in describing the steady growth of Soviet bases and activities on the continent and compares it to the recent shrinkage in the United States' presence. Surely the emergence of Soviet domination of the politics of Antarctica — which could happen in the next decade or so — will affect the working of the Treaty system as much as any legal requirements for the environment or the exploitation of minerals that the group may draw up? But the various authors in the Charney collection stick carefully to the question of the legal regimes for the environment, barely noting this issue.

Given the two different viewpoints in these books, how should the Treaty be judged? Will it sink under the weight of its new burden, namely the mineral resource issue? Certainly the vessel is frail, has 14 captains instead of one and the boiler goes out half of the time. But there is no other ship in sight; the alternatives to the Antarctic Treaty that have been advanced so far offer no practical plan for who will spend the money to run the stations, make weather forecasts, carry out communications in the difficult polar conditions and so on. So we might as well stay on board. It may help if a storm comes up. □

Deborah Shapley is Washington Editor of *Nature* and author of a forthcoming book, *The Seventh Continent: Antarctica in a Resource Age*.